FACTORS INFLUENCING THE SECRETION OF NITROGEN IN SHEEP SALIVA

1. THE DISTRIBUTION OF NITROGEN IN THE MIXED AND PAROTID SALIVA OF SHEEP

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SUMMARY.

Measurements were made on the distribution of nitrogen in the mixed and parotid salivary secretion of sheep and on the amount of nitrogen secreted daily in the parotid saliva.

The distribution of nitrogen in the mixed saliva followed the same pattern as that in the parotid secretions obtained when the glands were in a quiescent state. The concentration of nitrogen in both fluids depended on the rate of secretion.

Although the concentration of total nitrogen in the mixed and parotid saliva increased during short-term stimulation, the pattern of nitrogen distribution was not affected. During prolonged stimulation of the parotid glands the nitrogen composition of the saliva changed.

Quantitatively, urea was the most important nitrogen compound in both the mixed and parotid saliva, accounting for 60-70 p.c. of the total nitrogen.

The average volume of saliva secreted daily by one parotid gland was $2380 \pm 43{\cdot}8$ ml. and the average total nitrogen content was $295{\cdot}5 \pm 5{\cdot}77$ mg. when the nitrogen intake was 14·8 gm. per day.

Some implications of the amount and nature of the nitrogen compounds in relation to the ruminal metabolism of nitrogen in sheep are discussed.

INTRODUCTION.

Although salivary secretion in small laboratory animals has been extensively investigated, few corresponding studies have been made on ruminants. Scheunert and Trautmann (1921) concluded that only short-term studies could be conducted on sheep with permanent unilateral parotid fistulae, since the denial of the secretion from even one parotid gland resulted in nutritional failure and the animals developed mouth fistulae and finally died in a cachectic condition. Also, histological examination of the operated parotid glands showed them to be extensively fibrosed. McDougall (1948), studying the composition and output of sheep's saliva, also encountered difficulties in maintaining secretion from the parotid glands when temporary cannulae were put into the ducts. Other techniques were employed by this author to collect the saliva samples, but could be used only over limited periods.

More recently, Denton (1957) described the surgical preparation and post-operative management of a colony of sheep fitted with permanent unilateral parotid fistulae. Active secretion was maintained from the gland for 6 to 93 weeks and, providing the animals were given ample sodium each day, rumination and digestion did not appear to suffer. Prior to this work most of the investigations on the salivary secretions of ruminants gave more emphasis to the physical and chemical properties of the saliva than to the factors governing the rate of secretion.

With respect to chemical composition, McDougall (1948) reviewed the literature up to 1948, and himself presented a detailed analysis of the mixed and parotid saliva of sheep, particularly of their inorganic constituents. He showed that of the 1·0 to 1·4 gm. of dry matter per 100 ml. of saliva, nitrogen represented only 9 to 36 mg.

Other workers have also analysed ruminant saliva for its nitrogen content and as can be seen from the summary of these results below the level of nitrogen is extremely variable.

Author	Nature of Sample	Total-N (mg. p.c.)
Scheunert and Trautmann (1921)	Sheep – parotid	28-42
	Sheep – submaxillary	60-100
Trautmann and Albrecht (1931)	Goat – parotid	10-46
McDougall (1948)	Sheep – parotid	9-36
	Sheep – mixed	9-20
Denton (1957)	Sheep – parotid	10-30
Sharma (1936)	Buffalo – parotid	Trace

Neither the factors which control the level of nitrogen in the salivary secretions of ruminants, nor the form in which the nitrogen occurs, have so far been investigated, although the presence of urea was demonstrated by McDonald (1948). In view of the large volume of saliva secreted daily by ruminants (Colin, 1886; Markoff, 1913; Hydén, 1958) it appeared that the salivary nitrogen could make an important contribution to the rumen, and undergo the same processes of digestion and metabolism by the rumen organisms as sources of dietary nitrogen. It was therefore considered desirable to have information on the distribution of the salivary nitrogen and to delineate the factor(s) governing the amount of nitrogen in the salivary secretions. The results of such an investigation provide the material to be reported in this paper.

Materials and Methods.

Experimental animals and diet.

A group of mature merino wethers of similar age, weight and breeding was selected and fitted with rumen fistulae (Jarrett, 1948). Each animal was individually penned or

kept in a metabolism crate and maintained in the same position in the colony (see Denton, 1957b). A ration of oaten chaff, lucerne chaff, oat grain and commercial sheep cubes (see Moir and Somers, 1957) providing an intake of 680 gm. of dry matter and 14·8 gm. of nitrogen per day, was fed at 0900 hours. The diet proved palatable as evidenced by the fact that it was consumed within two hours of presentation.

Sampling techniques.

Samples of mixed saliva were collected into graduated cylinders as it dripped from the tongues of animals under sodium pentobarbitone sedation, induced and maintained by the intrajugular injection of Veterinary Nembutal (Abbott – 1 cc. containing 60 mg. of Nembutal in alcohol, 10 p.c., and propylene glycol 20 p.c.). The head was allowed to hang down over the end of the operating table so that the saliva dripped freely from the end of the tongue under gravity, while the jaws were held apart by inserting a rubber stopper between the dental pad and the incisors. Usually 10 to 15 ml. of anaesthetic were required to produce a period of sedation sufficient to permit a 30 min. collection after an initial flushing of the mouth with the saliva secreted over the first 5 to 6 minutes. Collections were made only during complete sedation as the first signs of returning consciousness were accompanied by an erratic flow.

Originally the 30 min. sample from any one animal was collected in three individual parts, each over a 10 min. interval, but as the nitrogen content of the separate sub-samples showed such close agreement subsequent chemical analyses were done on the pooled material. The frequency of sampling was limited to not more than once a week.

Quantitative collections of parotid saliva were made from four animals, each with a unilateral parotid duct cannulated, and a fifth animal with both ducts cannulated (see Somers, 1958) when their post-operative recovery was complete and they had resumed their normal eating pattern.

A further group of four animals, each fitted with permanent unilateral parotid fistulae (Denton, 1957) were used to collect samples of parotid saliva after a post-surgical period of 3 to 4 weeks.

In recognition of the range of stimuli known to affect the rate of salivary secretion (Comline and Kay, 1953; Coates *et al.*, 1956; Somers, 1957) attempts were made to standardise the sampling procedures. When samples of mixed or parotid saliva were collected over half-hourly periods the sheep were fasted for the previous 24 hours to reduce to a minimum mechanical stimulation by dietary roughage of the receptors in the throat and cardiac region of the oesophagus (Clark and Weiss, 1952). If rumination occurred during the short-term collections of parotid saliva the samples were discarded.

A strict feeding discipline was practised and an attempt was made to maintain a constant environment with the minimum of disturbance and handling of the animals at all times.

Except for the animal with both parotid ducts cannulated the operated groups received a ruminal infusion of 50 gm. of $NaHCO_3$ daily per cannulum.

Analytical methods.

The total nitrogen (T.N.) content of the saliva was determined by a micro-Kjeldahl method similar to that described by McKenzie and Wallace (1954), provision being made to allow slightly larger amounts of nitrogen to be determined. 0·01 N HCl was used for the titrations.

Ammonia nitrogen (NH_3-N) was estimated by the method of Conway (1950).

Urea nitrogen (U.N.) was determined by a modification of the micro-diffusion procedure used by Wu and Wu (1951) for human saliva. To obtain satisfactory recoveries of urea nitrogen the time of action of the urease phosphate preparation had to be increased

to 20 min. and a period of two hours allowed for the diffusion. A weaker solution of HCl (N/100) was employed for the titrations during which a stream of CO_2-free air was directed over the central well of the Conway unit. Corrections were applied for the nitrogen derived from sources other than urea.

Protein nitrogen. The "Biuret" test was used to determine whether the saliva contained protein. The violet shade which developed on application of this qualitative test was taken to signify the presence of a higher protein. As an alternative to precipitation methods which proved unsatisfactory for the quantitative estimation of the protein nitrogen, the samples of saliva were dialysed in cellophane tubing against a large volume of distilled water which was kept agitated and changed at intervals over a period of 30 hours. No further reduction in the amount of nitrogen remaining in the saliva took place by extending the dialysing time and it was shown that the samples were free of urea. Toluene was added as a preservative. The nitrogen content of the material remaining in the tubing, the non-dialysable nitrogen (N.D.N.), was determined by the method used for the determination of total nitrogen.

Residual nitrogen (R.N.). For convenience of expression that fraction of the dialysable nitrogen (D.N.) not accounted for by the urea nitrogen was termed residual nitrogen.

In the work to be reported in this and subsequent papers in the series chemical determinations were done in duplicate and unless the agreement between duplicates was $\pm$ 2 p.c. the determinations were repeated.

RESULTS.

The animals with the temporary parotid cannulae suffered no apparent discomfort from the presence of the cannulae which remained *in situ* from 3 to 5 weeks. They gained weight, maintained their normal eating habits, and showed no aversion to chewing on the operated side of the jaw. When a cannula was dislodged rapid healing followed, whereupon it was observed that the saliva once more entered the mouth.

To give some idea of the large day to day variability in the concentration of nitrogen in the mixed and parotid secretions, the data have been summarised in Table 1.

An explanation of the variability in the nitrogen concentration of the saliva was sought in terms of differences in the rate of secretion as it was observed that the rate of secretion remained relatively constant during any one collection but varied between samplings, even in the same animal.

Analysis of the data for each group of sheep showed that there was a highly significant correlation between the average rate of secretion and the concentration of nitrogen in the saliva: $r = 0{\cdot}9212$ ($P < 0{\cdot}001$) in the case of the mixed saliva, and $r = 0{\cdot}8901$ ($P < 0{\cdot}001$) in the case of the parotid saliva. The regression equations, $Y = -9{\cdot}19 + 13{\cdot}63x$, and $Y = -5{\cdot}34 + 24{\cdot}37x$, were also derived to express the relationships between the average rate of secretion (x), and the concentration of nitrogen (Y), in the mixed and parotid secretions, respectively. Figs. 1 and 2 show the relationships for the individual data in each group.

The distribution of the total nitrogen in 24 samples of the mixed and 60 samples of the parotid saliva was determined. In the presentation of the results

TABLE 1.

The concentration of nitrogen in the mixed and parotid salivary secretions of sheep.

	Mixed saliva					
	Days					
Sheep no.	1	2	3	4	5	6
1	14·4	12·2	18·6	9·4		
2	11·6	15·1	10·1	20·3		
3	12·0	8·7	17·6	9·1		
4	17·2	13·4	18·9	16·1		
5	9·2	21·3	16·8	12·7		
6	20·0	14·9	9·1	18·3		
7	17·5	10·2	21·1	12·9		
8	10·3	13·1	17·1	—		
9	21·1	12·2	11·9	10·3		
10	11·1	—	19·3	12·4		
11	18·8	9·8	—	13·1		
12	8·7	10·0	15·2	12·1		
	Parotid saliva					
13	17·7	13·5	9·8	10·4	15·1	16·4
14	10·0	18·9	14·0	11·5	13·9	9·4
15	12·5	17·3	22·7	13·8	9·7	16·9
16	8·6	14·7	10·0	15·4	12·4	19·5
17 (a)	18·9	14·6	9·2	16·9	10·5	12·1
17 (b)	10·6	13·1	12·6	8·9	14·8	19·3
18	12·3	18·7	10·4	19·2	14·0	11·3
19	12·0	12·2	13·7	11·7	21·2	15·5
20	20·1	9·8	15·9	13·2	8·1	10·3
21	9·1	16·2	10·0	11·7	19·0	12·5

Note: (i) Results are expressed in mg./100 ml. of saliva.
(ii) The prefix (a) or (b) in the case of sheep no. 17 refer to the left and right parotid glands, respectively.
(iii) Sheep nos. 18 to 21 had permanent parotid fistulae.

in Table 2 the samples have been grouped according to their total nitrogen concentration and the distribution of this nitrogen in the various fractions has been shown as a percentage of the total nitrogen.

No ammonia nitrogen could be detected in any of the samples, a finding which is consistent with that of Schmitz (1923) who analysed human saliva for ammonia. In the case of the human saliva, however, it was shown that ammonia is rapidly produced in the mouth by the action of the oral bacteria on the salivary urea.

It can be seen from Table 2 that there was a high degree of uniformity in the distribution of the nitrogen in the various fractions, irrespective of the concentration of total nitrogen. The pattern of distribution of the nitrogen in the mixed secretions accorded well with that in the parotid saliva, suggesting that the former were composed essentially of parotid saliva.

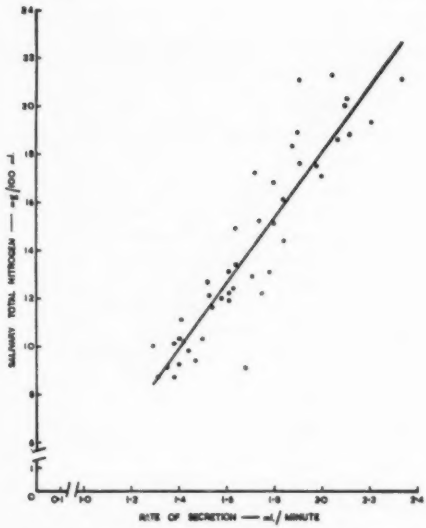

Fig. 1. Regression of the concentration of nitrogen in the mixed saliva of sheep on the rate of secretion.

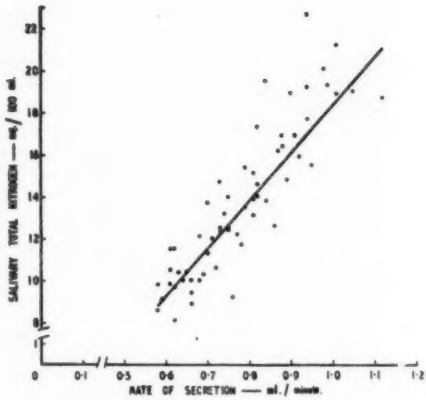

Fig. 2. Regression of the concentration of nitrogen in the parotid saliva of sheep on the rate of secretion.

TABLE 2.

The distribution of nitrogen in the mixed and parotid salivary secretions of sheep.

Range in total nitrogen mg./100 ml.	No. of samples	N.D.N. p.c.	D.N. p.c.	U.N. p.c.	R.N. p.c.
		Mixed saliva			
8-12	7	20·7 (17·6-25·2)	79·3 (74·8-82·4)	64·4 (62·1-68·0)	14·6 (6·8-20·0)
12-16	7	18·9 (11·1-24·6)	81·1 (75·4-88·9)	65·5 (62·3-71·9)	14·2 (11·6-18·0)
16-20	7	17·4 (13·5-19·7)	82·6 (80·3-86·5)	63·9 (61·1-67·1)	17·7 (13·1-21·0)
>20	3	19·2 (17·8-20·3)	80·8 (79·7-82·2)	62·6 (62·3-64·3)	17·9 (16·2-20·1)
		Parotid saliva			
8-12	21	17·4 (10·2-22·6)	82·7 (77·4-89·8)	64·6 (60·1-72·8)	18·1 (11·6-27·0)
12-16	23	18·8 (13·0-23·1)	81·2 (77·4-88·0)	65·1 (61·8-70·0)	16·2 (10·1-23·2)
16-20	13	19·4 (15·8-23·5)	80·5 (76·5-84·3)	65·4 (60·8-71·1)	15·2 (10·4-20·0)
>20	3	21·1 (15·2-24·2)	78·9 (75·8-84·8)	65·2 (61·5-68·7)	13·7 (10·6-16·1)

Note: The results have been expressed as a percentage of the total nitrogen.

Although the concentration of total nitrogen in both the mixed and parotid saliva increased with the rate of secretion, and thus with the level of glandular activity, the pattern of nitrogen distribution in the saliva did not change. It appeared, therefore, that irrespective of the level of glandular activity the mechanisms by which the nitrogenous compounds were transferred into the saliva were capable of maintaining a constancy in the nitrogen composition of both secretions.

To test this hypothesis further advantage was taken of the stimulus to the secretory rate induced by the presence of rumen fluid in the mouth to observe if any changes took place in the distribution of nitrogen during the higher rates of secretion, and therefore during increased activity of the salivary glands.

During the collection of the saliva under Nembutal anaesthesia it was observed that traces of ruminal fluid in the mouth augmented the flow of saliva as much as five- to sixfold. A similar response was elicited by touching the crown of the tongue with a swab just moistened with ruminal fluid or acetic acid (see also Coates *et al.*, 1956; Somers, 1957; Denton, 1957). Mineral acids and sodium acetate failed to produce a similar response. A typical response to rumen liquor is shown in Fig. 3.

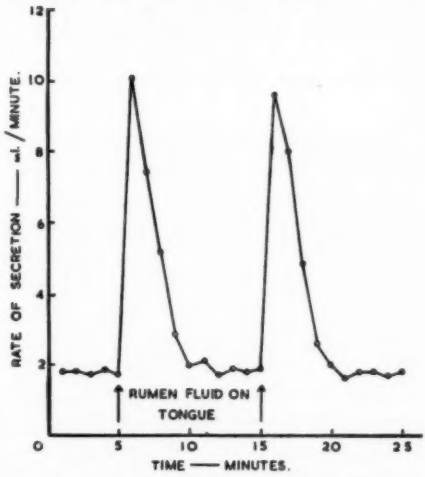

Fig. 3. The effect on the secretion rate of the mixed saliva of sheep in response to rumen fluid on the tongue.

Samples of saliva were collected separately, before the stimulus and over the following 4-minute interval. Table 3 shows the results of the distribution of nitrogen in the two samples for 6 sheep, where the total nitrogen is shown in mg. per 100 ml. and the remainder of the results as a percentage of the total nitrogen.

In each instance the concentration of total nitrogen in the saliva produced by stimulation was approximately doubled but the distribution pattern of this nitrogen was not altered to any appreciable extent as can be seen in Table 3. Because the percentage of nitrogen in the respective fractions of the unstimulated and stimulated saliva remained the same, despite the increased activity of the glands, it appeared that the transfer of nitrogen from the glands into the saliva depended on the rate at which water crossed the surfaces of the secreting glandular epithelium. However, it will be seen in the following section, dealing with the secretions from the parotid glands, that protracted stimulation did cause a change in the distribution of nitrogen in the saliva.

TABLE 3.

A comparison of the nitrogen distribution in the stimulated and unstimulated salivary secretions of sheep.

Sheep no.	Nature of sample	T.N. mg./100 ml.	N.D.N. p.c.	D.N. p.c.	U.N. p.c.	R.N. p.c.
1	Unstimulated	16·5	19·6	80·4	64·1	16·3
	Stimulated	22·9	18·5	81·5	63·3	18·2
2	Unstimulated	12·8	15·2	84·9	61·9	22·9
	Stimulated	28·0	17·5	82·5	68·1	14·3
3	Unstimulated	10·9	18·8	81·2	65·0	16·2
	Stimulated	25·6	17·1	83·0	62·8	20·2
4	Unstimulated	15·2	22·2	77·8	67·4	10·4
	Stimulated	29·2	18·4	81·7	64·5	17·2
5	Unstimulated	19·3	21·1	78·9	67·0	11·9
	Stimulated	31·5	20·3	79·7	65·2	14·5
6	Unstimulated	12·7	19·1	80·9	63·8	17·1
	Stimulated	26·4	16·0	84·0	64·2	19·8

With the continuous secretion of the parotid glands it was possible to examine to what extent the influence of natural stimulation by feeding affected the nitrogen composition of the saliva. It had been observed that the rate of secretion could increase as much as tenfold over the preceding basal rate for periods of 1 to 2 min. when eating commenced, whereas increases of up to five- to sixfold were not uncommon for periods of 30 to 45 min.

Initially observations were made on the nitrogen components in the saliva by feeding small amounts of lucerne chaff to create a stimulus extending up to 5 min. Notwithstanding the increased rate of secretion and concentration of total nitrogen, the distribution of nitrogen was not changed. However, when the feeding period was extended to 30 to 50 min. by presenting larger amounts of the lucerne chaff, changes did occur in the percentage of nitrogen in the various salivary fractions as seen in Table 4.

The substantial increase in the concentration of nitrogen in the saliva which accompanied the higher rates of secretion caused by the feeding stimulus was attended by a change in the nitrogen distribution pattern. As shown in Table 4 there was a reduction in the percentage of nitrogen in the non-dialysable fraction in each instance. The increased proportion of nitrogen in the dialysable fraction was accounted for mainly by an increased proportion of nitrogen in the residual fraction, the ratio of urea nitrogen to total nitrogen remaining relatively constant. Such alterations in the pattern of nitrogen distribution resulting from prolonged stimulation of the parotid glands apparently involve changes in the cellular physiology whereby the rate of transfer of the smallest molecular nitrogen components into the saliva can be increased with a corresponding reduction in the rate of secretion of the larger molecular nitrogen components. The observation that short-term stimulation did not cause changes in the pattern of nitrogen distribution of either the mixed or parotid saliva despite the increased concentration of nitrogen, indicates that the duration over

TABLE 4.

Changes in the distribution of nitrogen in the parotid saliva of sheep due to stimulation.

Sheep no.	Nature of sample	Vol. of saliva (ml.)	Collection time (min.)	Total-N (mg./100 ml.)	N.D.N.	D.N.	U.N.	R.N.
1	Pre-feeding	28·2	30	17·7	16·5	83·5	65·4	20·0
	Feeding	237·6	33	32·2	8·1	91·9	68·4	23·5
2	Pre-feeding	19·8	30	9·4	20·1	79·9	64·4	15·5
	Feeding	317·4	46	23·1	6·7	93·3	67·2	26·1
3	Pre-feeding	21·9	30	12·5	18·3	81·7	62·8	18·9
	Feeding	324·0	40	27·8	5·4	94·7	73·1	21·6
4	Pre-feeding	23·7	30	15·4	15·4	75·7	65·4	10·3
	Feeding	288·7	36	26·3	3·6	96·4	68·5	28·9
5	Pre-feeding	31·6	30	19·0	12·7	87·3	73·5	13·9
	Feeding	384·8	52	33·2	9·3	90·7	70·3	20·5
6	Pre-feeding	22·2	30	13·2	21·6	78·4	67·8	10·6
	Feeding	369·8	43	24·8	10·1	89·9	66·8	23·2

Note: The results for the various nitrogen fractions are expressed as a percentage of the total nitrogen.

which the stimulus operates is an important factor in determining the nitrogen composition of the saliva.

During the course of the investigations on the parotid secretions, some preliminary observations were made on the amount of nitrogen secreted from a single gland in 24 hours. Daily recordings were made on each of 6 sheep fed the same ration over a 4-day period. The same diet was used in each case since Denton (1956) has shown that the volume of saliva produced daily varies with the diet. Table 5 shows the daily volume and nitrogen content of the saliva produced from one parotid gland in each sheep.

With a mean daily volume of 2,388 ml. (S.D. of the mean ± 43·8) the nitrogen output from the single parotid gland was 295·5 mg. (S.D. of the mean 5·8). These results are in very good agreement with those previously recorded by the author (Somers, 1958) using an animal with both parotid ducts cannulated.

TABLE 5.

The daily volume and nitrogen content of the parotid saliva of sheep.

Sheep no.	Average daily volume (ml.)	Average nitrogen content (mg.)
1	2300 (1975-2590)	282 (251-327)
2	2527 (2379-2633)	287 (273-310)
3	2654 (1990-2600)	305 (272-365)
4	2429 (2178-2675)	284 (263-309)
5	2318 (2041-2560)	313 (286-344)
6	2353 (2068-2549)	301 (273-340)

DISCUSSION.

The results of this investigation are in agreement with those of previous workers (Colin, 1886; Eckhard, 1893; Ellenberger and Hofmeister, 1887; Scheunert and Trautmann, 1921; Babichev *et al.*, 1930; Trautmann and Albrecht, 1931; Sharma, 1936; and others) who have observed that the parotid glands of ruminants secrete continuously. Colin (1886) considers that the sublingual glands also secrete continuously, while Scheunert and Krzywanek (1929) believe that a similar function can be attributed to the ventral portion of the buccal glands in sheep. However, the mandibular glands function only during the consumption of feed (Colin, 1886; Scheunert and Trautmann, 1921) and not during rest or rumination.

Recently, Hydén (1958) estimated that these continuous salivary secretions produce a total flow of 6 to 12 l. per day in sheep. On the basis of the daily output of saliva from a single parotid gland (McDougall, 1948; Denton, 1956; Coates *et al.*, 1956; Somers, 1958; Stewart and Dougherty, 1958) it is clear that the parotid secretions make the largest contribution to the total flow. This is supported by the close agreement in the nitrogen distribution in the mixed and parotid saliva found in these investigations.

With respect to the nitrogen components in the saliva, the most outstanding feature is the high proportion of urea nitrogen, which accounts for some 60 to 70 p.c. of the total nitrogen. It seems probable that the salivary urea is responsible for part of the ammonia normally found in the ruminal fluid, particularly as it can be inferred from the work of Pearson and Smith (1943) that urease activity in the rumen is high throughout the day.

As it is now well established that the rumen bacteria can use dietary urea for the synthesis of their own cellular protein it is logical to assume that salivary urea can be used in the same way thus enabling some recovery of a nitrogenous substance normally considered entirely a waste product. In this way a nutritional function can be attributed to salivary urea.

Failure to find a satisfactory protein precipitant for the saliva suggested that the material belonged to the glycoprotein class. The analysis of the mixed secretions, and the parotid saliva secreted by the animals in a quiescent state, showed that the non-dialysable nitrogen only accounted for some 15 to 20 p.c. of the total nitrogen. Under prolonged stimulation brought about by feeding, the level of non-dialysable nitrogen fell to 5 to 10 p.c. of the total nitrogen.

Anrep (1920-21) has shown that the mucin nitrogen (protein nitrogen) secreted in the submaxillary saliva of dogs decreases to zero in response to long-term artificial stimulation. His results demonstrated further that the mucin nitrogen in the saliva came from the mucin or mucinogen stored in the gland while the non-mucin nitrogen was derived from body fluids. During stimulation no mucin formation took place in the gland. A similar situation in the case of the parotid secretions of sheep could explain our observations on the reduction in the non-dialysable nitrogen under extended stimulation.

The dialysable nitrogen in the mixed saliva and the parotid saliva collected from quiescent animals accounted for some 75 to 80 p.c. of the total nitrogen. However, stimulation of the parotid secretion caused an increase in the percentage of dialysable nitrogen to 90 to 95 p.c. of the total nitrogen. On the basis of these experiments it seems that the excretion of the dialysable material proceeds independently of the protein nitrogen (N.D.N.). Such changes in the nitrogen composition of the saliva reflect differences in the delivery of water and small molecular nitrogen components to the gland, and their rate of transfer across the surfaces of the secretory cells.

In view of the wide range of stimuli known to affect the rate of secretion from the parotid glands (Somers, 1957; Denton, 1957), and the high correlation between the total nitrogen and the rate of secretion over sampling periods of short duration, it was surprising to find that the variability in the total nitrogen and volume of saliva secreted in 24 hours was so small. The fact that the animals were fed the same ration to which they had been accustomed for two to three months previously, and which they had been trained to eat in two hours, coupled with a strict routine in handling, feeding and collecting by the one attendant to minimise psychic stimulation, seemingly contributed to the low level of variability observed.

Since the salivary nitrogen could be of significance in supplying part of the nitrogen requirement of the rumen organisms, especially during maintenance and sub-maintenance levels of nitrogen intake, experiments were designed to determine the influence of the level of nitrogen intake on the amount of nitrogen secreted in the saliva. The findings are reported in the following paper.

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REFERENCES.

Anrep, G. V. (1920-21): J. Physiol., 54, p. 319.
Babichev, G. A., Perstnev, N. S., and Kulesko, I. I. (1930): Russian physiol. J., 13, p. 636.
Clark, R., and Weiss, K. E. (1952): J. South Afric. Vet. Med. Ass., 23, p. 162.
Coates, D. A., Denton, D. A., Goding, J. R., and Wright, R. D. (1956): J. Physiol., 131, p. 13.
Colin, G. C. (1886): Traité de physiologie comparée des animaux, Vol. 1, 3rd Ed., Paris.
Comline, D. S., and Kay, R. N. B. (1955): J. Physiol., 129, p. 55P.
Conway, E. J. (1950): Microdiffusion Analysis and Volumetric Error. 3rd Ed., London. Crosby, Lockwood & Son.
Denton, D. A. (1956): J. Physiol., 131, p. 516.
Denton, D. A. (1957): Quart. J. exp. Physiol., 42, p. 72.
Denton, D. A. (1957b): Nature, 179, p. 341.
Eckhard, C. (1893): Centralblatt für Physiologie, 7, p. 365.
Ellenberger, W., and Hofmeister, V. (1887): Arch. Anat. Physiol. Lpz. Suppl., 138.
Hydén, S. (1958): Ann. Roy. agr. Coll. Sweden, 24, p. 55.
Jarrett, I. G. (1948): J. C.S.I.R., 21, p. 311.

Markoff, J. (1913): Biochem. Z., 57, p. 1.
McDonald, I. (1948): Thesis, University of Cambridge.
McDougall, E. I. (1948): Biochem. J., 43, p. 99.
McKenzie, H. A., and Wallace, H. S. (1954): Aust. J. Chem., 7, p. 55.
Moir, R. J., and Somers, M. (1957): Aust. J. ag. Res., 8, p. 253.
Pearson, R. M., and Smith, J. A. B. (1943): Biochem. J., 37, p. 148.
Scheunert, A., and Trautmann, A. (1921): Pflug. Arch. ges. Physiol., 182, p. 33.
Scheunert, A., and Krzywanek, Fr. W. (1929): *Ibid.*, 223, p. 472.
Schmitz, H. W. (1923): J. Lab. clin. Med., 8, p. 78.
Sharma, G. K. (1936): Indian J. vet. Sci., 6, p. 266.
Somers, M. (1957): Aust. vet. J., 33, p. 297.
Somers, M. (1958): Nature, 182, p. 400.
Stewart, W. E., and Dougherty, R. W. (1958): J. anim. Sci., 17, p. 1220.
Trautmann, A., and Albrecht, H. (1931): Arch. Wiss. Prakt. Tierheilk., 64, p. 93.
Wu, D. Y., and Wu, H. (1951): Proc. Soc. exp. Biol. Med., 76, p. 130.

FACTORS INFLUENCING THE SECRETION OF NITROGEN IN SHEEP SALIVA

2. THE INFLUENCE OF NITROGEN INTAKE UPON BLOOD UREA NITROGEN AND UPON THE TOTAL NITROGEN AND UREA NITROGEN IN THE PAROTID SALIVA OF SHEEP

by M. SOMERS

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SUMMARY.

Urea was added to the rumen of three merino sheep at the rates of 5, 10 and 20 gm. daily to increase the overall nitrogen intakes from 10·7 gm. in the basal ration to 13·0, 15·4 and 20·0 gm. per day.

A marked increase in the total amount of nitrogen secreted in parotid saliva was found to occur as the nitrogen intake of the sheep rose. A similar high correlation was found between the level of nitrogen intake and the amount of urea nitrogen secreted in the saliva daily.

Between the highest and the lowest levels of nitrogen intake the magnitude of the increases were 0·26 gm. of total nitrogen and 0·18 gm. of urea nitrogen per gland per day.

The concentration of urea nitrogen in the blood increased with the level of nitrogen intake of the sheep, but fluctuations in the level of blood urea could not be accounted for by nitrogen intake alone.

A significant correlation was found between the concentrations of urea nitrogen in the blood and in the saliva. This offered a partial explanation of the higher concentrations of salivary urea resulting from the increased nitrogen intakes.

INTRODUCTION.

Although physical and technical limitations preclude the simultaneous collection of saliva from the individual glands in the conscious sheep and therefore quantitative information on the total salivary flow recent techniques have been devised for the collection of the continuous secretions from the parotid glands (Denton, 1957; Somers, 1958). These techniques have permitted long-term studies on the parotid secretions of sheep displaying apparently normal physiological reactions.

With respect to the total salivary flow, Somers (1961) provided presumptive evidence to indicate that the parotid secretions make the largest contribution. This worker also determined the nitrogen content of the parotid flow to be approximately 0·3 gm. per gland per day when the nitrogen intake of the

sheep was constant (see also Somers, 1958). Further, nitrogen distribution studies on mixed and parotid saliva showed that urea was quantitatively the most important nitrogen compound, accounting for 60-70 p.c. of the total nitrogen.

As the salivary nitrogen compounds become exposed to the same microbial digestive and metabolic processes in the rumen as dietary nitrogen compounds, it became important to know whether, and to what extent, salivary nitrogen secretion is affected by varying intakes of nitrogen in the diet. The work reported in this paper deals with an investigation into the amounts of nitrogen and urea secreted daily in the parotid saliva of sheep fed a basal ration to which graded levels of urea were added via the rumen. By this means it was possible to observe the influence of an increasing nitrogen intake on the parotid secretions uncomplicated by changes in the bulk or energy status of the diet which have been shown to affect, or which might affect, the production of saliva by the parotid glands (Ellenberger and Hofmeister, 1887; Block, 1939; Denton, 1956).

During the course of these investigations concurrent studies were also made to determine the influence of nitrogen intake on blood urea nitrogen and, in turn, the influence of the latter on the concentration of urea nitrogen in the saliva.

Materials and Methods.

Experimental animals and diet.

Three 3-year-old merino wethers were selected for the experiments on the basis of weight uniformity (39 to 41 kg. body weight), but more particularly on their general acquiescence to handling. Each sheep was fitted with a permanent rumen fistula (Jarrett, 1948).

A surgical technique similar to that described by Denton (1957) was used for explanting the parotid duct and papilla into a stab incision in the cheek. After healing was complete the parotid duct was cannulated via the exteriorised orifice with pliable polythene tubing of appropriate diameter and of sufficient length to allow the saliva to drip into graduated cylinders positioned clear of the metabolism crates in which the animals were housed. The cannulae were held in position by passing the external end through a hole in a plastic disc then attached to the cheek by means of loose stay sutures, care being taken to see that no tension was set up on the cannulae and to see that the jaw movements on the operated side were not impaired. The animals were maintained thus for one month before the quantitative collections of saliva were commenced.

In this pre-experimental period the animals were conditioned to being housed in individual metabolism crates, which were segregated from the rest of the sheep colony, and to the basal ration to be used. Over the same period each animal was trained to eat the entire daily ration within two hours.

The basal ration consisted of 350 gm. oaten chaff, 50 gm. lucerne chaff, 50 gm. oats and 150 gm. of commercial sheep cubes (see Moir and Somers, 1957). Each daily ration contained 600 gm. of dry matter and 10·7 gm. of nitrogen and was fed at 0900 hours.

Tap water was freely available at all times, and each animal was given a daily ruminal infusion of 50 gm. of $NaHCO_3$ to replace the sodium lost in the saliva.

Throughout the investigation a strict daily routine in feeding, sampling and administration of the $NaHCO_3$ was adhered to so that the sheep were handled as little as possible and with the minimum of disturbance at all times.

Treatments and sampling procedure.

The feeding of the basal ration alone constituted treatment 1 which lasted for seven days. During this time recordings were made of the volume of saliva produced daily by the parotid glands of the individual sheep, sufficient material being kept for chemical analysis. In treatments 2, 3 and 4 the basal ration was also fed and in addition, 5, 10 and 20 gm. of urea were added to the rumen so that the nitrogen intake for these respective treatments was 13·0, 15·4 and 20·0 gm. per day. The urea infusions were made daily over the time interval corresponding with the eating of the basal ration, the sheep receiving each new treatment for three days before the seven-day collection period commenced.

The order of the treatments was arranged so that the animals were receiving an increasing level of nitrogen throughout the investigation.

Toluene was added to the collecting cylinders as a preservative during the collection of the saliva.

Samples of venous blood were obtained from the jugular vein on the final three days of each collection period for blood urea nitrogen determinations. These samples were taken four hours after feeding using heparin as an anti-coagulant.

Chemical methods.

Salivary total nitrogen and urea nitrogen were determined by the same methods as those employed previously (Somers, 1961).

Blood urea nitrogen was estimated in accordance with the method outlined by the manufacturers of the Urease-Dunning preparation (Hynson, Westcott and Dunning, Inc., Baltimore) in an all-glass aeration apparatus. However, boric acid was used to absorb the ammonia released during aeration and 0·01 N HCl for the titrations. Corrections were made for the ammonia arising from sources other than urea.

RESULTS.

The pertinent data showing the means and the daily range for the three sheep on the four respective treatments have been summarised in Table 1.

Daily volumes of saliva.

Although the volume of saliva varied from 1·89 to 3·01 litres per day over the eighty-four observations (3 sheep for 4 periods of 7 days) the range in the mean daily volume for the three sheep in each of the four periods was only from 2·27 to 2·42 litres per day. An analysis of variance was carried out on the data which showed that the differences between sheep and treatments and days were not significant.

With respect to the volume of saliva secreted daily, Denton (1956) has shown that the volume of saliva produced by the parotid glands of sheep is relatively constant on any one diet but varies with changes in the composition of the diet, while Ellenberger and Hofmeister (1887) and Block (1939) have found that the physical properties of the diet have an important bearing on the amount of saliva secreted by the parotid glands of ruminants.

In this investigation the overall nitrogen intake was increased by adding graded levels of urea directly to the rumen. Therefore, there were no changes in the amount of food nor the composition of the food the sheep actually con-

TABLE 1.

Mean values and the range in the volume of saliva; the concentration of total and urea nitrogen in the saliva; the total amount of nitrogen and urea nitrogen in the saliva and the concentration of blood urea nitrogen in relation to the daily nitrogen intake.

Treatment	Nitrogen intake gm./day	Sheep no.	Volume of saliva l./day	Saliva				Blood
				Total nitrogen		Urea nitrogen		
				mg. p.c.	mg./day	mg. p.c.	mg./day	Urea nitrogen mg. p.c.
1	10·7	1	2·38 (2·20-2·63)	9·9 (8·4-11·9)	235 (197-273)	6·7 (5·5-8·4)	160 (133-192)	11·1 (9·2-13·8)
		2	2·27 (1·98-2·74)	7·8 (5·8-9·8)	175 (148-201)	5·3 (4·0-6·5)	119 (103-134)	10·8 (8·7-13·5)
		3	2·32 (1·94-2·72)	7·9 (5·4-9·4)	181 (141-214)	5·4 (3·6-6·7)	122 (95-142)	9·6 (7·6-11·8)
2	13·0	1	2·33 (2·15-2·62)	11·4 (8·8-13·2)	264 (230-288)	7·9 (5·8-9·6)	182 (152-212)	14·4 (12·0-17·3)
		2	2·38 (1·98-2·83)	10·2 (6·8-12·2)	239 (194-282)	6·8 (5·4-9·0)	157 (119-178)	11·4 (9·4-14·3)
		3	2·35 (1·92-2·95)	11·2 (6·6-13·7)	258 (196-308)	7·6 (4·1-9·6)	173 (121-208)	12·9 (10·1-16·2)
3	15·4	1	2·38 (2·12-2·86)	13·5 (9·7-15·7)	317 (276-352)	9·2 (6·2-11·2)	216 (178-247)	21·6 (18·8-25·3)
		2	2·35 (2·08-2·94)	12·7 (9·2-14·7)	294 (260-329)	8·5 (5·8-10·0)	196 (171-220)	18·6 (16·4-21·6)
		3	2·40 (1·97-3·01)	12·6 (7·9-15·9)	295 (239-329)	8·8 (5·4-11·7)	205 (163-242)	20·7 (18·3-23·7)
4	20·0	1	2·42 (2·10-2·93)	20·1 (14·6-25·1)	478 (429-543)	13·8 (10·4-17·0)	328 (281-396)	26·2 (21·8-30·5)
		2	2·36 (1·89-2·81)	19·2 (13·4-24·3)	442 (376-499)	12·7 (9·7-16·1)	293 (253-344)	22·7 (18·9-26·5)
		3	2·40 (2·00-2·87)	19·0 (17·4-20·4)	458 (376-556)	13·0 (10·9-14·4)	316 (242-395)	26·0 (22·5-29·4)

sumed, and this appears to account for the consistency in the observed results on the daily volumes.

On the basis of these findings it was possible to examine the influence of increasing the nitrogen intake on the salivary nitrogen excretion, uncomplicated by the factor of volume.

Salivary nitrogen data.

The mean values for the concentration of total nitrogen in the saliva, the amount of nitrogen secreted daily by a single parotid gland in each of the three sheep, and the salivary urea nitrogen data are given in Table 1.

Both the total nitrogen and the urea nitrogen secreted in the saliva daily were significantly correlated with the level of nitrogen intake, the respective correlation coefficients being 0·9940 and 0·9943. The regression equation,

$$Y = -112{\cdot}175 + 28{\cdot}113x,$$

was derived to express the relationship between the daily secretion of total nitrogen in the saliva (Y) and the nitrogen intake (x). Similarly, a regression equation, $Y = -77{\cdot}810 + 19{\cdot}193x$, was derived to express the relationship between the salivary urea nitrogen (Y), and the nitrogen intake (x). These relationships are shown in Fig. 1 where each point represents the mean of twenty-one values.

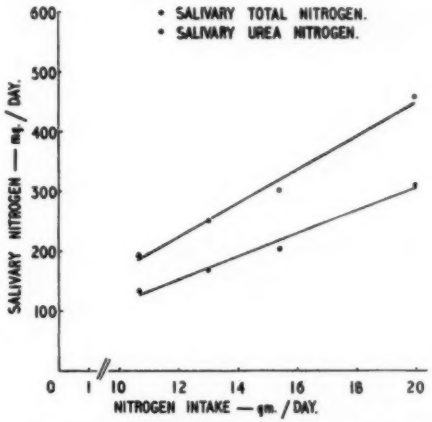

Fig. 1. Regression of (a) salivary total nitrogen (mg. per day) and (b) salivary urea nitrogen (mg. per day) on nitrogen intake (gm. per day).

Results from the analysis of variance of the total nitrogen in the saliva (mg. per day) and from the urea nitrogen in the saliva (mg. per day) are given in Table 2.

The sheep differences were significant at the 1 p.c. level in the case of the total nitrogen and urea nitrogen secreted in the saliva daily. The regressions between each of these factors and the nitrogen intake were highly significant ($P < 0{\cdot}001$) emphasising the importance of the level of nitrogen fed to the sheep on the total nitrogen and the urea nitrogen content of the parotid secretions.

In the case of the regression between the urea nitrogen in the saliva and nitrogen intake the deviations from the regression did not reach the 5 p.c. level of significance. On the other hand, the deviations from the regression of total nitrogen in the saliva on nitrogen intake were significant; indicating

TABLE 2.

Analysis of variance: total nitrogen and urea nitrogen in the saliva.

Source of variation	Total nitrogen in saliva			Urea nitrogen in saliva		
	Degrees of freedom	Mean square	F	Degrees of freedom	Mean square	F
Sheep	2	9456·68	6·71**	2	6371·64	7·19**
Treatment	3	267019·85	189·41***	3	124199·57	140·23***
Regression	1	791450·61	164·73**	1	368358·91	415·91***
Deviations from regression	2	4804·47	3·41*	2	2119·90	2·39 NS
Sheep × treatment	6	921·72	<1 NS	6	410·21	<1 NS
Error	72	1409·73		72	885·67	

NS Not significant.
* Significant at 5 p.c. level.
** Significant at 1 p.c. level.
*** Significant at 0·1 p.c. level.
Note: Where the term "treatment" is used it refers to the levels of nitrogen intake.

that a simple linear regression does not fully describe the relationship. However, the proportion of the total variability between treatments accounted for by the linear regression was 98·8 p.c. Thus, for the purposes of this investigation the fitting of a complex response curve was not considered warranted.

Blood and salivary urea nitrogen data.

The pertinent results for the concentration of urea nitrogen in both the saliva and blood have been summarised in Table 1. An analysis of variance was carried out on each set of data in which it was found that the sheep differences and the sheep by treatment interactions were not significant in either instance. However, both the blood and salivary urea nitrogen concentrations were significantly influenced ($P < 0{\cdot}001$) by the amounts of nitrogen the sheep received.

As the nitrogen intake was increased by the infusion of urea into the rumen there was a corresponding increase in the concentration of blood urea nitrogen, the correlation coefficient between the two components being 0·9723 ($P < 0{\cdot}05$). From the regression analysis of the concentration of blood urea nitrogen (Y), on nitrogen intake (x), the equation, $Y = -6{\cdot}823 + 1{\cdot}625x$, was derived. This relationship has been presented in Fig. 2 along with that between the concentration of urea nitrogen in the saliva and the nitrogen intake which were positively correlated, $r = 0{\cdot}9992$ ($P < 0{\cdot}01$). The data for the concentration of urea nitrogen in the saliva (Y), and the nitrogen intake (x), were subjected to regression analysis to obtain the regression equation, $Y = -2{\cdot}744 + 0{\cdot}812x$.

In Fig. 2 the respective relationships between the concentration of urea nitrogen in blood and in saliva on nitrogen intake have been shown in the form of simple linear regressions. While the deviations from the regression of the concentration of salivary urea nitrogen on nitrogen intake were not significant, those for the regression of the concentration of blood urea nitrogen on nitrogen intake were significant at the 5 p.c. level. However, the regression did account for 94·5 p.c. of the variability between treatments.

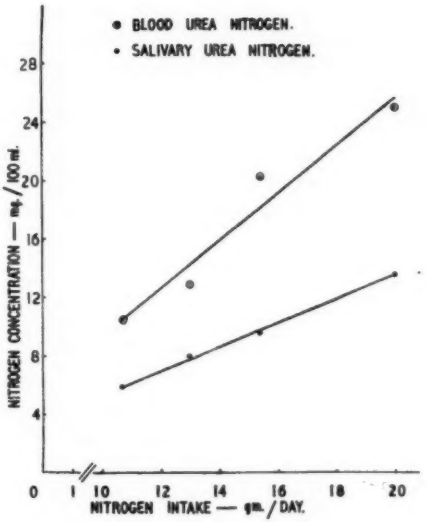

Fig. 2. Regression of (a) blood urea nitrogen (mg. per 100 ml.) and (b) salivary urea nitrogen (mg. per 100 ml.) on nitrogen intake (gm. per day.).

Although the nitrogen intake significantly influenced the concentration of urea nitrogen in the blood the deviations from the regression between these two variables indicated that factors other than the nitrogen intake were involved in determining the observed fluctuations in the concentration of urea nitrogen in the blood.

The dependency of the concentration of the salivary urea nitrogen on the concentration of urea nitrogen in the blood was indicated by the significant correlation coefficient ($r = 0{\cdot}9625$). A significant regression was obtained by analysing the data for the concentration of salivary urea nitrogen (Y), and the concentration of blood urea nitrogen (x), from which the equation, $Y = +1{\cdot}208 + 0{\cdot}468x$ was developed. The relationship between these two components has been shown in Fig. 3.

In addition to the significance of the regression, however, the deviations from the regression were also significant. An analysis of covariance of the salivary urea nitrogen concentration and blood urea nitrogen concentration showed that there were highly significant differences between the salivary urea nitrogen means after they had been adjusted for variability in the blood urea nitrogen ($F = 18{\cdot}24$ on 3 and 23 degrees of freedom ($P < 0{\cdot}001$)). These findings indicated that the concentration of urea nitrogen in the saliva was governed by some factor(s) other than the concentration of urea nitrogen in the blood.

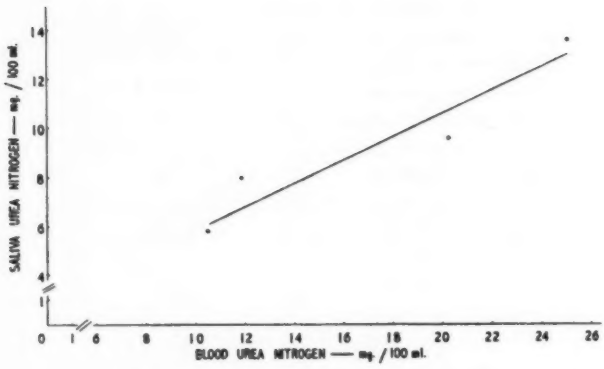

Fig. 3. Regression of salivary urea nitrogen (mg. per 100 ml.) on blood urea nitrogen (mg. per 100 ml.).

DISCUSSION

The results of this investigation clearly demonstrated that as the overall nitrogen intake was increased there was a correspondingly larger amount of nitrogen secreted in the parotid saliva. As the nitrogen intake was increased from 10·7 to 20·0 gm. per day, the average increase in the amount of nitrogen secreted was 0·26 gm. per day for a single parotid gland. This constituted a recovery of 5·6 p.c. of the additional 9·3 gm. of nitrogen added to the rumen.

While a large proportion of the increases in the nitrogen secreted daily in the saliva could be attributed to the additional nitrogen intake, the latter was not the only factor involved in determining the amount of nitrogen secreted in the saliva. Thus, the daily salivary total nitrogen was not a simple linear function of nitrogen intake but the amount of daily salivary urea nitrogen was related to the nitrogen intake in a linear manner, indicating that the increases in the salivary urea could be directly attributed to the level of urea added to the rumen.

The high proportion of the total salivary nitrogen accounted for by urea nitrogen found in earlier studies (Somers, 1957, 1961) was again confirmed. Although there is very little information available on the factors that influence the levels of salivary urea Lewis (1957) demonstrated a qualitative relationship between the concentration of urea in the mixed salivary secretions of sheep and the concentration of rumen ammonia. Simonnet *et al.* (1957), on the other hand, showed that the intra-jugular injection of a solution of urea (0·2 gm. per cc.) into sheep, at the rate of 0·2 gm. per kg. body weight, caused a fairly rapid increase in the secretion of urea in the mixed salivary secretions. The significance of these results is difficult to interpret as they were not related to the volume of saliva produced.

In this investigation a significant regression between the concentration of blood urea nitrogen and salivary urea nitrogen was found, but at no stage did the latter exceed the former and no simple relationship between these two factors was evident. It seems probable that the observed increases in the blood urea concentration could have been due to the additions of urea to the rumen, and not necessarily to the increases in the overall nitrogen intake, since Dinning *et al.* (1948), and Repp *et al.* (1955), have both observed that the concentration of blood urea increased when urea and other non-protein nitrogen compounds were administered to ruminants.

Lewis (1957) studied the fluctuations in the blood urea concentration in relation to the nitrogen metabolism of sheep, and found them to be better correlated with different rumen ammonia concentrations than with the overall level of nitrogen intake. Since the production of ammonia in the rumen influences the level of blood urea, which in turn influences the urea excretion by the parotid glands as shown in this investigation, further information is necessary on the relationship between ammonia production in the rumen and urea excretion in the saliva of sheep. The results of a study of this relationship are reported in the following paper.

REFERENCES.

Block, E. L. (1939): Sechenov J. Physiol., U.S.S.R., 27, p. 200.
Denton, D. A. (1956): J. Physiol., 131, p. 516.
Denton, D. A. (1957): Quart. J. exp. Physiol., 42, p. 72.
Dinning, J. S., Briggs, H. M., Gallup, D. W., Orr, H. W., and Butler, R. (1948): Amer. J. Physiol., 153, p. 41.
Ellenberger, W., and Hofmeister, V. (1887): Arch. Anat. Physiol. Lpz. (Suppl.), 138, p. 147.
Jarrett, I. G. (1948): J. C.S.I.R., 21, p. 311.
Lewis, D. (1957): J. Agr. Sci., 48, p. 438.
Moir, R. J., and Somers, M. (1957): Aust. J. agr. Res., 8, p. 253.
Repp, W. W., Hale, W. H., Cheng, E. W., and Burroughs, W. (1955): J. anim. Sci., 14, p. 118.
Simonnet, H., le Bars, H., and Molle, J. (1957): Com. Rend. Acad. Sci., Paris, 244, p. 943.
Somers, M. (1957): Aust. vet. J., 33, p. 297.
Somers, M. (1958): Nature, 182, p. 400.
Somers, M. (1961): Aust. J. exp. Biol., 39, p. 111.

FACTORS INFLUENCING THE SECRETION OF NITROGEN IN SHEEP SALIVA

3. FACTORS AFFECTING THE NITROGEN FRACTIONS IN THE PAROTID SALIVA OF SHEEP WITH SPECIAL REFERENCE TO THE INFLUENCE OF AMMONIA PRODUCTION IN THE RUMEN AND FLUCTUATIONS IN LEVEL OF BLOOD UREA

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SUMMARY.

Quantitative measurements were made on the volume of saliva secreted hourly by the parotid glands of merino sheep over a period of 24 hours on three separate days. At the same time fluctuations in the concentration of urea and total nitrogen in the saliva were studied in relation to the production of ammonia in the rumen and changes in the level of blood urea that occurred throughout the day.

Both the rumen ammonia and the blood urea levels followed a relatively simple cyclical pattern over each 24-hour period. However, two distinct phases were apparent in the salivary nitrogen cycle. The first, a short phase, followed the stimulation changes associated with eating. The second phase was prolonged and more complex. It was influenced by changes in the level of blood urea, but additional factors also operated in the second phase.

Changes in the second phase of the salivary nitrogen cycle have been discussed in relation to fluctuations in the concentration of rumen ammonia and blood urea.

INTRODUCTION.

McDonald (1948) presented an outline of the main pathways of nitrogen metabolism in the rumen of sheep, and produced experimental evidence to support his hypothesis that a nitrogen cycle is established as a result of the normal digestive processes in the rumen. He demonstrated that ammonia may be directly absorbed into the venous blood leaving the rumen (see also Bouckaert and Oyaert, 1952; Chalmers *et al.*, 1954; and Lewis *et al.*, 1957) and that ammonia is absent, or present only in extremely low concentrations, in the peripheral blood. On the basis of these observations he postulated that the absorbed ammonia undergoes metabolism in the liver and that the main end-product entering into the general circulation for excretion is urea. Since this worker also demonstrated the presence of urea in the mixed salivary secretions of sheep, he pointed out that part of the urea formed in the liver may enter the rumen via these secretions and thus complete the nitrogen cycle.

Two lines of evidence are now available to support McDonald's original suggestion that the ammonia absorbed from the rumen is converted to urea in the liver. Lewis (1957) provided presumptive evidence when he showed that the blood urea concentration could be correlated with different rumen ammonia concentrations. More direct evidence of the conversion of ammonia to urea in the liver was obtained by Lewis *et al.* (1957) who showed that, as the concentration of ammonia in the portal blood increased, so did the concentration of urea in the systemic circulation. These workers also examined the efficiency of the liver in removing ammonia from the portal flow, and found that, although leakage of ammonia through the liver into the systemic circulation occurred at relatively low concentrations, there was no significant increase in the arterial ammonia concentration until the portal blood ammonia concentration exceeded approximately 0·8 m. moles per litre.

Independent observations have confirmed the presence of urea in the salivary secretions of sheep (Somers, 1957; Lewis, 1957; and Simonnet *et al.*, 1957). Further, Lewis (1957) showed that there is a qualitative relationship between the levels of ammonia produced in the rumen and the concentration of urea in the mixed salivary secretions of sheep, while Simonnet *et al.* (1957) observed an increase in the concentration of urea in the mixed secretions in response to intravenous injections of urea. Similar observations have been made by Somers (1961c) in response to urea injections.

Investigations by the author (Somers, 1961b) showed that the amount of total nitrogen and urea nitrogen secreted in the parotid saliva daily increased significantly when graded levels of urea were added to the rumen to augment the nitrogen intake from the diet. Some of the observed increase in the salivary urea could be attributed to the elevated levels of blood urea.

Insofar as the production of ammonia in the rumen influences the level of blood urea, which in turn influences the urea secretion by the parotid glands, further experiments were designed to study more closely the interdependence of and interrelationships between the respective phases in the nitrogen cycle. Information was also obtained on the extent to which the parotid glands secrete nitrogen, including urea, in the saliva. The results provide the subject matter of this paper.

Materials and Methods.

Experimental animals and diet.

Three mature merino wethers of the same age, weight and breeding were used in the experiments. Each sheep was fitted with a permanent rumen fistula (Jarrett, 1948), an exteriorised loop of the carotid artery and a permanent unilateral parotid fistula (Denton, 1957). The animals were conditioned to the metabolism crates in which they were kept during the experiments and were accustomed to the manipulations required for collecting samples of blood and rumen contents.

In the three-week pre-experimental period the animals were trained to eat the entire ration offered daily within one hour of presentation. The daily ration consisted of 350 gm.

of oaten chaff, 30 gm. of lucerne chaff, 100 gm. of oat grain and 200 gm. of commercial sheep cubes (see Moir and Somers, 1957) providing an intake of 14·8 gm. of nitrogen and 680 gm. of dry matter per day. This ration was chosen because of its palatability and because preliminary investigations showed that it gave rise to relatively high concentrations of ammonia in the rumen. Feeding was limited to once daily, at 0900 hours, but the sheep had free access to water at all times.

Except on the days when samples of blood and ruminal material were collected each animal received a ruminal infusion of 50 gm. of sodium bicarbonate commenced with the presentation of food and extending over the following 2 hours. In this regard, Denton (1956) has found that sheep with permanent unilateral parotid fistulae may be maintained in good health indefinitely, provided that they receive sufficient sodium bicarbonate to compensate for the sodium lost each day in the large volume of saliva secreted by the parotid glands.

Sampling procedures and techniques.

Sampling was carried out over a 24-hour period on three days, each separated by an interval of six days. On each of the sampling days, ruminal material was collected hourly for ammonia nitrogen analyses. The material was obtained by applying mild suction to a tube inserted into the rumen via the rumen cannulae. The volume of saliva secreted over each hour was also measured, and kept as a separate sample for nitrogen analyses. The technique used for making the quantitative collection of the parotid saliva has been described previously (Somers, 1961b).

To avoid undue stress to the animals, blood samples were obtained from the carotid loop only on the first and last days of sampling. These samples were taken directly into a 5 ml. syringe at two-hourly intervals using heparin as an anticoagulant.

Analytical methods.

After filtering the samples of rumen contents through bolting silk (grade 2xx, Henry Simons, Sydney), ammonia nitrogen levels were estimated on the filtrate by the method of Conway (1950), the results being expressed as milligrams of ammonia nitrogen per 100 ml. of rumen fluid.

Blood urea nitrogen was determined by the Conway method, the urease-phosphate preparation being made in accordance with the formula described by Wu and Wu (1951), and using Urease-Dunning tablets (Hynson, Westcott and Dunning, Inc., Baltimore). The urease-phosphate was allowed to act on the blood in the outer chamber of the Conway unit for 20 minutes prior to the addition of alkali and after a further hour the liberated ammonia was titrated. Controls were also set up in order to apply corrections for the nitrogen arising from sources other than urea.

Methods for determining total nitrogen and urea nitrogen have been described previously (see Somers, 1961a).

RESULTS.

Preliminary statistical analyses of the data indicated that the most suitable method for handling the results was to consider the days as triplicate observations in the case of salivary nitrogen and rumen ammonia nitrogen concentrations, and duplicate observations in the case of the blood urea nitrogen concentrations. That is, the differences between days and the interactions of days with sheep and times of sampling were negligible compared with the main effects of hours and sheep.

To give adequate mathematical expression to the trends in the various nitrogen concentrations in the rumen, blood and saliva with time, it was necessary to omit the results obtained at and up to 1000 hours for each day of sampling. During this period, i.e. when the sheep were consuming their daily ration, variations in the volume of saliva secreted and the total nitrogen and urea nitrogen concentrations in the saliva were such that no satisfactory statistical expression could be found to include the results as part of the whole daily cycle. The nature of the changes which occurred in the volume and salivary nitrogen components will be discussed separately below.

Further, since it was much more convenient to fit curves to equally spaced data and as blood samples were obtained for urea nitrogen analyses only at two-hourly intervals, the results for the even-hour collections were deleted. This was done, however, only when it was certain that the deletion of these results did not significantly alter the basic nature of the individual curves. Thus, the analyses of variance were prepared on 108 observations, namely, three sheep by three days by 12 sampling times, except in the case of the blood urea nitrogen concentrations, where 72 observations were used, namely, three sheep by two days by 12 sampling times. Days were regarded as replicate observations. Table 1 shows the mean value for each of the nitrogen factors investigated and the mean volume of saliva secreted in the preceding hour.

TABLE 1.

Summary of the mean values of the main experimental results.

Hour of sampling	Blood urea nitrogen mg. per 100 ml.	Salivary total nitrogen mg. per 100 ml.	Salivary urea nitrogen mg. per 100 ml.	Rumen ammonia nitrogen mg. per 100 ml.	Volume of saliva ml.
1100	19·6	12·7	8·5	15·3	79·9
1300	21·8	19·7	13·6	24·9	115·2
1500	25·2	25·5	17·8	18·0	117·3
1700	27·6	23·0	16·5	13·4	120·8
1900	26·7	17·1	12·2	10·7	120·0
2100	24·2	13·5	9·6	8·2	122·8
2300	22·8	12·2	8·3	7·2	118·6
0100	21·9	11·5	7·8	5·7	99·6
0300	21·2	10·6	7·2	4·8	87·4
0500	20·9	10·7	7·1	4·5	74·7
0700	20·5	11·8	8·0	4·6	81·9
0900	20·4	12·1	8·3	5·5	98·9

The analysis of variance and the regression equation for each of the separate factors investigated have been presented in Appendix A. For each of the factors the variability between sampling times comprised the largest proportion of the total variability and the sums of squares of deviations due to the sampling times, on 11 degrees of freedom, were partitioned into that due to regression and that due to the deviations from the regression. The fitted curves, Fig. 1, are harmonics, and the order of the harmonic in each case was determined so that the residual was not significant (cf. error variance).

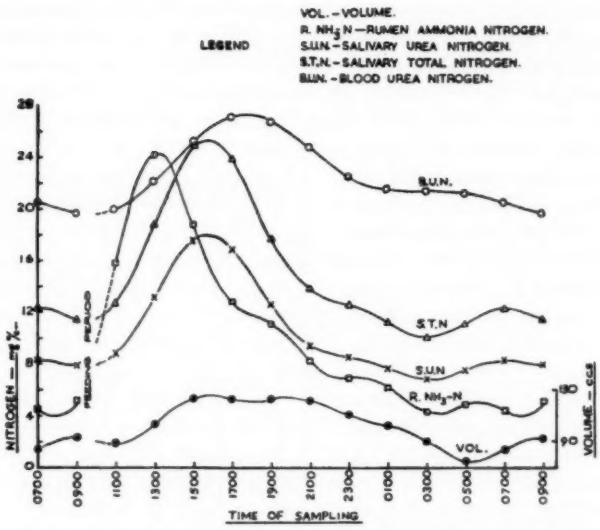

Fig. 1. Fitted harmonic curves showing diurnal fluctuations in the concentration of total and urea nitrogen in the parotid saliva of sheep and their relationship to changes in (a) rumen ammonia nitrogen, (b) blood urea nitrogen, and (c) the volume of saliva secreted.

With respect to the regression equations in Appendix A the angle θ in degrees for any given time is determined by the equation:

$$\theta = 15(x - 10) \text{ where } x \text{ is the hour of collection.}$$

The time at which the maximum nitrogen concentration occurred for each factor was obtained by differentiating the regression curves and equating the differentials to zero. The same was done for the volume of saliva secreted hourly. Table 2 shows the maximum nitrogen concentration attained in each of the components, together with the times at which these maxima were reached. It also includes the standard errors of estimate.

TABLE 2.

Summary of calculated maxima and times at which maxima were attained in the nitrogen concentrations and in the volume of saliva secreted together with the standard errors of estimate.

	Rumen ammonia nitrogen	Blood urea nitrogen	Salivary total nitrogen	Salivary urea nitrogen	Saliva volume (ml.)
Maximum concentration (mg. per 100 ml.)	24·2	27·3	25·3	17·9	125·7
Time of maxima (hour)	13·05	17·45	15·45	15·45	15·40
Standard errors of estimate	1·9083	1·2913	1·6696	1·1634	16·1667

As mentioned previously, variations occurred in the data relating to the salivary secretions during the feeding period and no satisfactory expression could be found to include this data as part of fitted curves, as shown in Fig. 1. It was apparent that the dynamics of secretion during the feeding period were appreciably different from those operating over the remainder of the day in regard to the volume of saliva secreted, and the concentration of total nitrogen and urea nitrogen in the saliva. To give an indication of the nature of the fluctuations which took place in salivary data during the feeding period, and to show the relationship between these fluctuations and those which occurred during the post-feeding interval, a typical set of observations have been shown in Fig. 2 for a six-hour period.

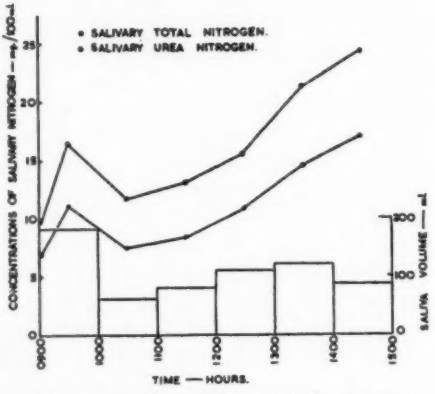
• SALIVARY TOTAL NITROGEN.
• SALIVARY UREA NITROGEN.
CONCENTRATIONS OF SALIVARY NITROGEN — mg/100 ml
SALIVA VOLUME — ml
TIME — HOURS.

Fig. 2. Feeding and post-feeding changes in the volume of parotid saliva secreted hourly and the concentration of total nitrogen and urea nitrogen in the saliva. Feeding occurred only during the first hour, i.e. 0900 to 1000 hours.

It can be seen from Fig. 2 that the volume of saliva secreted over the first hour, when the animal was eating, was substantially larger than that collected over the succeeding hourly periods. Also, the concentration of total nitrogen and urea nitrogen in the saliva was higher than that in a sample taken just prior to feeding, or that in the saliva collected in the first and second hours after feeding.

DISCUSSION.

The results of this investigation clearly demonstrate the cyclical and interdependent nature of the trends that occur in the concentration of rumen ammonia nitrogen, blood urea nitrogen, and salivary total and urea nitrogen, as a result of a large single daily feed. However, from the results two distinct phases are apparent in the salivary cycle. The first occurs as an immediate response to the act of eating, when both the volume of saliva secreted and the respective nitrogen concentrations in the saliva are affected. These observations are in accord with those previously made by the writer (Somers, 1961a) where a relationship between the concentration of total nitrogen in the saliva and the rate of secretion was found. That this relationship did not maintain throughout the day can be seen from Fig. 1. This response is, nevertheless, transitory and the second phase, which constitutes the major daily cycle, is then revealed.

Consequent upon feeding, the concentration of ammonia nitrogen in the rumen fluid increased at a relatively rapid rate, reaching a maximum of 24 mg. per 100 ml. four hours after the presentation of food. Thereafter it declined so that the final concentration was very similar to that at the commencement of the 24-hour sampling period.

Evidence that part of the ammonia produced in the rumen was absorbed, metabolised to urea in the liver, and the urea subsequently discharged into the systemic circulation, is indicated by the rise in the concentration of blood urea nitrogen which took place. This rise in the blood urea nitrogen was progressive, and continued until the maximum concentration of 27 mg. per 100 ml. of blood occurred 8¾ hours after feeding. This was approximately five hours after the concentration of rumen ammonia nitrogen had reached its peak. In this regard, Lewis (1957) has shown that changes in the blood urea nitrogen concentration are primarily a reflection of the ammonia production in the rumen. Nevertheless, it is possible that urea arising from products of normal protein metabolism also contributed to the observed increase in the blood urea nitrogen concentration. Phillipson (1952) demonstrated that the flow of ingesta from the abomasum is relatively constant. However, with a single feed consumed in a short period of time, as in the work at present under discussion, the pattern of flow from the rumen may vary and the maintenance of a high level of blood urea may consequently be a reflection of plethora conditions at the intestinal level superimposed upon the urea level derived from rumen ammonia.

Whereas it might have been expected that changes in the concentration of urea nitrogen in the saliva would follow closely those in the blood, this was the case for only part of the 24-hour cycle. Following feeding, the concentration of urea nitrogen in both the blood and the saliva increased, and continued to do so for the next seven hours. However, the rate of increase in the concentration of urea nitrogen in the saliva exceeded that in the blood. Clearly, the salivary urea nitrogen secretion rate is limited by the blood urea nitrogen level. Furthermore, these data show a similar limiting concentration as occurred in another experiment (Somers, 1961c), where a concentration limit in the salivary urea was observed over a larger scale of blood urea nitrogen concentrations. Notwithstanding the fact that the urea nitrogen in the blood continued to rise for a further two hours before reaching the maximum concentration, the concentration of salivary urea nitrogen and total nitrogen started to decline after the seventh hour, and continued to decline thereafter, although a slight upward trend in the final two hours was observed.

Reference to Fig. 1 makes it apparent that the relationship between the concentration of blood urea nitrogen and the salivary urea nitrogen throughout the day is not a simple one, and that the concentration of urea nitrogen in the saliva cannot be explained merely in terms of passive diffusion of urea from the blood into the parotid secretions. Nor can the salivary nitrogen concentrations be satisfactorily explained in terms of differences in the hourly rate of secretion of the saliva over the whole 24-hour period.

While there appeared to be an association between the concentration of total nitrogen and urea nitrogen in the parotid secretions and the volume of saliva secreted hourly over the first seven hours, each reaching a maximum at approximately 1600 hours, the association between these factors did not follow the same pattern for the rest of the day. For example, when the minimum hourly volume of saliva was secreted, the total nitrogen and urea nitrogen concentrations in the saliva were low, but were not at their minima. It is apparent, therefore, that factors additional to the hourly rate of secretion of the saliva and the concentration of urea nitrogen in the blood, operate in determining the concentration of salivary urea nitrogen at various times during the day.

Somers (1961a) showed that the concentration of nitrogen in the mixed and parotid secretions of fasted sheep was related to the rate of secretion with both resting and stimulated glands. The same situation certainly obtained during the feeding period in this investigation, as can be seen from Fig. 2. Further, data obtained from sheep under anaesthesia showed that the rate of secretion of mixed saliva increased as did the nitrogen concentration of the saliva, when a swab just moistened with rumen fluid was placed on the crown of the tongue for a brief period (Somers, 1961a). Acetic acid is known to elicit a similar response with respect to the rate of secretion (Coates *et al.*, 1956).

The greater hourly volume of saliva secreted during the afternoon, and up to approximately 2100 hours, could well be associated with rumination which has been reported to stimulate salivary production (McDougall, 1948; Denton, 1957).

Inspection of the results of Scheunert and Trautmann (1921) shows that there is essentially no change in the percentage of nitrogen in the parotid saliva secreted by sheep during rest, rumination and feeding. However, observations by the author (unpublished data) indicate that there is usually a rise in the concentration of salivary total nitrogen and urea nitrogen associated with the increased rate of secretion during rumination. If, therefore, the secretion of saliva follows the same stimulation trend during rumination as that due to eating, the curves shown in Fig. 1 for the total salivary nitrogen and salivary urea nitrogen are composites of the influence of both volume changes and blood urea changes.

During the immediate pre- and post-feeding hours only minor fluctuations occurred in the concentration of blood urea nitrogen. Therefore, the respective data for each of these two hours provided a means of estimating the influence of changes in the hourly rate of secretion *per se* on the concentration of urea nitrogen in the saliva. In the hour immediately prior to feeding, the mean volume of saliva secreted was 99 ml. and it had a mean urea nitrogen concentration of 8·3 mg. per 100 ml. When the animals were eating their daily ration in the following hour and the glands were stimulated by the act of eating, the mean hourly volume rose to 160 ml. and the urea nitrogen concentration of this saliva increased to 11·8 mg. per 100 ml. Assuming that the augmented

rate of flow of saliva during rumination is attended by an increase in the concentration of urea nitrogen in the saliva similar in magnitude to that during eating, an increase of this order alone does not provide adequate explanation of the high concentration of salivary urea nitrogen and total nitrogen that occurred at 1545 hours. At that time the concentrations of urea nitrogen and total nitrogen in the saliva were far in excess of the concentrations observed in the feeding stimulated saliva. It is apparent, therefore, that the elevated blood urea level was responsible to a considerable degree for the high salivary urea nitrogen concentration at 1545 hours.

REFERENCES.

Bouckaert, J. H., and Oyaert, W. (1952): Zootechnia, 1, p. 21.
Chalmers, M. I., Cuthbertson, D. P., and Synge, R. L. M. (1954): J. agric. Sci., 44, p. 254.
Coates, D. A., Denton, D. A., Goding, J. R., and Wright, R. D. (1956): J. Physiol., 131, p. 13.
Conway, E. J. (1950): Microdiffusion Analysis and Volumetric Error. 3rd ed. London. Crosby, Lockwood and Son.
Denton, D. A. (1956): J. Physiol., 135, p. 227.
Denton, D. A. (1957): Quart. J. exp. Physiol., 42, p. 72.
Jarrett, I. G. (1948): J. C.S.I.R., 21, p. 311.
Lewis, D. (1957): J. agr. Sci., 48, p. 438.
Lewis, D., Hill, K. J., and Annison, E. F. (1957): Biochem. J., 66, p. 587.
McDonald, I. (1948): Thesis, University of Cambridge.
McDougall, E. I. (1948): Biochem. J., 43, p. 99.
McKenzie, H. A., and Wallace, H. S. (1954): Aust. J. Chem., 7, p. 55.
Moir, R. J., and Somers, M. (1957): Aust. J. ag. Res., 8, p. 253.
Phillipson, A. T. (1952): J. Physiol., 116, p. 84.
Scheunert, A., and Trautmann, A. (1921): Pflug. Arch. ges. Physiol., 192, p. 33.
Simonnet, H., le Bars, H., and Molle, J. (1957): Com. Rendus Acad. Sci., Paris, 244, p. 943.
Somers, M. (1957): Aust. Vet. J., 33, p. 297.
Somers, M. (1961a): Aust. J. exp. Biol., 39, p. 111.
Somers, M. (1961b): *Ibid.*, 39, p. 123.
Somers, M. (1961c): *Ibid.*, 39, p. 145.
Wu, D. Y., and Wu, H. (1951): Proc Soc. exp. Biol. Med., 76, p. 130.

APPENDIX A

Although the analysis of variance and the regression equation for each of the respective factors shown below were calculated from the data using ten significant figures, for the purposes of presentation the statistical results have been rounded and only three places of decimal have been retained.

In the analyses of variance the following symbols have been used to denote the levels of significance:

N.S. – Not significant.
* – Significant at the 5·0 p.c. level.
** – Significant at the 1·0 p.c. level.
*** – Significant at the 0·1 p.c. level.

(i) *Rumen ammonia nitrogen (mg. per 100 ml.) analysis of variance:*

Source of variation	Degrees of freedom	Sums of squares	Mean square	Variance ratio
Sheep	2	10·800	5·400	1·48 N.S.
Hours	11	4146·395	376·945	103·51***
Regression	8	4119·632	514·954	141·40***
x_1	1	2638·137		724·41***
x_2	1	366·249		100·57***
x_3	1	734·827		201·78***
x_4	1	22·669		6·22*
x_5	1	150·285		41·27***
x_6	1	106·406		29·22***
x_7	1	16·436		4·51*
x_8	1	84·625		23·24***
Deviations from regression	3	26·763	8·921	2·45 N.S.
Error	94	342·329	3·642	
Total	107	4499·524		

Regression equation:

$$Y = 10\cdot243 + 6\cdot990 \sin\theta + 2\cdot604 \cos\theta + 3\cdot689 \sin 2\theta - 0\cdot648 \cos 2\theta + 1\cdot668 \sin 3\theta - 1\cdot404 \cos 3\theta + 0\cdot552 \sin 4\theta - 1\cdot252 \cos 4\theta$$

(ii) *Blood urea nitrogen (mg. per 100 ml.) analysis of variance:*

Source of variation	Degrees of freedom	Sums of squares	Mean square	Variance ratio
Sheep	2	665·022	332·511	199·40***
Hours	11	452·232	41·112	24·65***
Regression	4	440·160	110·040	65·99***
x_1	1	216·530		129·85***
x_2	1	142·634		85·53***
x_3	1	32·395		19·43***
x_4	1	48·602		29·15***
Deviations from regression	7	12·071	1·724	1·03 N.S.
Error	58	96·720	1·668	
Total	71	1213·973		

Regression Equation:

$$Y = 22\cdot740 + 2\cdot453 \sin\theta - 1\cdot991 \cos\theta - 0\cdot949 \sin 2\theta - 1\cdot162 \cos 2\theta$$

(iii) *Saliva: total nitrogen (mg. per 100 ml.) analysis of variance:*

Source of variation	Degrees of freedom	Sums of squares	Mean square	Variance ratio
Sheep	2	119·872	59·936	21·50***
Hours	11	2560·736	232·794	83·52***
Regression	4	2530·444	632·611	226·95***
x_1	1	1977·721		709·52***
x_4	1	420·017		150·68***
x_5	1	103·253		37·04***
x_6	1	29·453		10·57**
Deviation from regression	7	30·292	4·327	1·55 N.S.
Error	94	262·016	2·787	
Total	107	2942·625		

Regression equation:

$$Y = 15{\cdot}030 + 6{\cdot}052 \sin\theta - 2{\cdot}789 \cos 2\theta - 1{\cdot}383 \sin 3\theta - 0{\cdot}739 \cos 3\theta$$

(iv) *Saliva: urea nitrogen (mg. per 100 ml.) analysis of variance:*

Source of variation	Degrees of freedom	Sums of squares	Mean square	Variance ratio
Sheep	2	67·303	33·651	24·86***
Hours	11	1374·841	124·986	92·34***
Regression	4	1364·236	341·059	251·97***
x_1	1	1078·104		796·49***
x_4	1	221·551		163·68***
x_5	1	52·780		38·99***
x_6	1	11·801		8·72**
Deviations from regression	7	10·605	1·515	1·12 N.S.
Error	94	127·235	1·354	
Total	107	1569·379		

Regression equation:

$$Y = 10{\cdot}410 + 4{\cdot}468 \sin\theta - 2{\cdot}026 \cos 2\theta - 0{\cdot}989 \sin 3\theta - 0{\cdot}467 \cos 3\theta$$

(v) *Saliva: volume (ml. per hour) analysis of variance:*

Source of variation	Degrees of freedom	Sums of squares	Mean square	Variance ratio
Sheep	2	312·796	156·398	<1 N.S.
Hours	11	33093·435	3008·494	11·51***
Regression	4	29615·667	7403·917	28·33***
x_1	1	17574·240		67·24***
x_2	1	9871·070		37·77***
x_5	1	978·009		3·74 N.S.
x_7	1	1192·347		4·56*
Deviations from regression	7	3477·768	496·824	1·90 N.S.
Error	94	24568·093	261·363	
Total	107	57974·324		

Regression equation:

$$Y = 103{\cdot}157 + 18{\cdot}041 \sin\theta - 13{\cdot}521 \cos\theta - 4{\cdot}256 \sin 3\theta - 4{\cdot}699 \sin 4\theta.$$

FACTORS INFLUENCING THE SECRETION OF NITROGEN IN SHEEP SALIVA

4. THE INFLUENCE OF INJECTED UREA ON THE QUANTITATIVE RECOVERY OF UREA IN THE PAROTID SALIVA AND THE URINARY EXCRETIONS OF SHEEP

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(*Accepted for publication 9th February, 1961.*)

SUMMARY.

Differential responses occurred in the amount of urea nitrogen and total nitrogen secreted in the parotid saliva, according to the nitrogen status of the sheep, when 1·4 gm. of urea nitrogen was injected into the blood daily. Of this amount of nitrogen, 0·1 gm. could be recovered in the secretion from one parotid gland daily when the sheep were in positive nitrogen balance, and 0·3 gm. when the sheep were in negative nitrogen balance.

The urinary excretion accounted for 1·0 gm. of the 1·4 gm. of urea nitrogen injected daily when the sheep were in positive nitrogen balance, but only 0·3 gm. per day at the lower nitrogen status.

The implications of these findings were discussed in relation to a urea conservation mechanism in sheep.

INTRODUCTION.

Schmidt-Nielsen *et al.* (1957) found that when the need for protein was high, such as in periods of rapid growth, or when the protein intake was low, the camel can conserve nitrogen by reducing the urinary excretion of urea to extremely low levels. The same workers showed that urea injected intravenously during periods of low nitrogen intake was not excreted in the urine, but was retained. Although there is no evidence to indicate that the camel or other ruminants can use urea directly for the synthesis of tissue proteins or other substances, there is abundant evidence to suggest that the microbial flora in the rumen do utilise urea for protein synthesis. Therefore, the only justification for a urea retention by ruminants would seem to depend on mechanisms being available for the transport of the urea from the blood back into the rumen. The salivary secretions of sheep have been shown to contain urea (McDonald, 1948; Somers, 1957; Lewis, 1957; Simonnet *et al.*, 1957), and since the secretion of saliva is continuous, this constitutes a pathway for the transport of urea into the rumen.

Although little information is available on the amount of urea flowing into the rumen via the saliva, or on the factors influencing the secretion of urea in the saliva, Lewis (1957) demonstrated a qualitative relationship between the levels of ammonia produced in the rumen and the concentration of urea in the mixed salivary secretions of sheep. On the other hand, Simonnet *et al.* (1957) observed an increase in the concentration of urea in the mixed saliva of sheep in response to intravenous injections of urea. Investigations by the author (Somers, 1961b) showed that there was a significant increase in the amount of total nitrogen and urea nitrogen secreted in the parotid saliva daily, when the overall nitrogen intake of the sheep was increased by adding graded levels of urea to the rumen. Part of the observed increase in the salivary urea could be attributed to the elevated levels of blood urea.

In view of these findings, and the fact that the secretion of saliva from the parotid and other of the salivary glands (see Colin, 1886; and Scheunert and Krzywanek, 1929) is a continuous process in ruminants, it appeared that the salivary secretions might provide an important pathway for the transport of blood urea and other nitrogen compounds back into the rumen, and in this way add to the supply of nitrogen available for microbial assimilation.

Further experiments were therefore designed to study the factors influencing the salivary nitrogen secretion in sheep. Data from short-term experiments made under anaesthesia on the amount of total nitrogen and urea nitrogen secreted by the parotid glands of sheep in response to urea injections are reported. However, the work in this paper is primarily concerned with the quantitative assessment of the amount of total nitrogen and of urea nitrogen secreted in the parotid saliva and the recovery of urea injected into the blood in these secretions, during periods of high and low nitrogen intake. Further, information was obtained on the extent to which the injected urea could be recovered in the urine or retained by the animal.

Experimental.

Experimental animals and diets.

Three mature merino wethers, each fitted with a permanent rumen fistula (Jarrett, 1948), an exteriorised loop of the carotid artery and a permanent unilateral parotid fistula (Denton, 1957) were used for the main experiments. One other animal with cannulae tied into both parotid ducts (see Somers, 1958) was used for short-term investigations. The animals were housed as a separate community in individual metabolism cages designed for the collection of excreta for balance studies. Each day the sheep were given a ruminal infusion of 50 gm. of $NaHCO_3$ to avoid various grades of sodium depletion (see Denton, 1957). This infusion commenced with the presentation of food and extended over the following two hours. Tap water was freely available at all times, and the sheep were fed once daily at 0900 hours. The diets used in the balance experiments were compounded from oaten and lucerne chaff, oat grain, and commercial sheep cubes (see Moir and Somers, 1957). The composition of the two diets used, one to provide a high level of nitrogen intake and the other a low level of nitrogen intake is shown below. Each diet was also designed to provide an equal amount of dry matter daily.

Composition of daily diets. Dry basis.

Diet	Oaten chaff gm.	Lucerne chaff gm.	Oat grain gm.	Sheep cubes gm.	Dry matter gm.	Total nitrogen gm.
1	350	30	100	200	680	14·53
2	500	30	150	—	680	6·76

Sampling procedures and treatments.

For short-term experiments under anaesthesia sedation was induced by the intravenous injection of Veterinary Nembutal (Abbott; 1 ml. containing 60 mg. Nembutal in 10 p.c. alcohol and 20 p.c. propylene glycol) and, after tracheal intubation, continued with a gas mixture of cyclopropane and oxygen in a closed circuit. The sheep suffered no apparent physiological stress under these conditions for periods of up to seven hours.

Prior to any collections being made on the conscious sheep, the animals were fed the experimental diet for at least two weeks. The initial eight-day collection period on each diet was used to determine the nitrogen balances of the sheep, and measure the daily secretion of total nitrogen and urea nitrogen in the saliva. This was followed by a further period of eight days, when each sheep received, in addition to the diet, a daily injection of 3·0 gm. of urea. The urea solution (0·2 gm. per ml.) was injected at the rate of 1·5 ml. per minute into the carotid artery. These injections were carried out with the presentation of food and were made with the minimum of disturbance to the animals. In most cases they continued to eat while the infusions were being made.

The saliva, urine and faeces were collected and measured at 0900 hours daily, the faeces being treated in the manner described by Moir and Williams (1950) and analysed at the end of each collection interval. Urine and saliva samples were analysed for total nitrogen and urea nitrogen daily. The technique for making the quantitative collection of the parotid saliva has been previously described by the writer (Somers, 1961b).

In the short-term experiments where samples of blood were taken, these were withdrawn from the jugular vein, using heparin as an anticoagulant. During these experiments samples of rumen contents were obtained directly through the rumen fistula.

Toluene was added to the vessels used to collect the urine and saliva.

Analytical methods.

The total salivary nitrogen was determined by an adaptation of the method of McKenzie and Wallace (1954) and the salivary urea nitrogen by a modification of the procedure described by Wu and Wu (1951). Urea nitrogen in the urine and blood was estimated by the Conway method (1950), as already described (Somers, 1961c).

Urine and faeces samples were analysed for total nitrogen in the following way: duplicate samples were digested according to the procedure of Willits *et al.* (1949), after which the digestion mixture was made up to 600 ml. with glass distilled water. An aliquot (20 ml.) of the solution was then alkali-steam distilled in a Parnas Wagner still, the ammonia being trapped in 4 p.c. boric acid, and titrated with 0·01 N HCl.

Ammonia nitrogen levels were obtained for rumen contents by the Conway method after the material had been filtered through bolting silk (grade 2xx, Henry Simons, Sydney), and the results were expressed as milligrams of ammonia nitrogen per 100 ml. of rumen fluid.

RESULTS.

Short-term experiments on the influence of adding urea to the blood of sheep upon the salivary urea secretion.

A number of experiments were carried out on sheep maintained under anaesthesia for periods of up to seven hours to determine the capacity of the parotid glands to secrete urea injected into the blood stream. The animals were denied food for 24 hours prior to the commencement of the experiment to reduce to a minimum any changes likely to occur in the blood urea concentration arising from the absorption of ammonia from the rumen (see Lewis, 1957). Direct determinations of the rumen ammonia nitrogen levels showed them to be low and relatively constant throughout the experiments. Urea nitrogen determinations on blood samples taken at the beginning and end of the first hour, which served as a control period, were found to be virtually the same in any one animal. During the control period the volume of saliva secreted over each 15-minute interval was analysed for both total and urea nitrogen.

After the first hours of observation each sheep was given an intracarotid infusion of 3·0 gm. of urea (0·2 gm. urea per ml.) over a period of 15 minutes. Samples of blood and rumen contents were collected at half-hourly intervals over the next six hours and the saliva secreted over each half-hour interval was kept as a separate sample for nitrogen analyses.

The results of a typical experiment made on a sheep with a permanent unilateral parotid fistula are shown in Fig. 1.

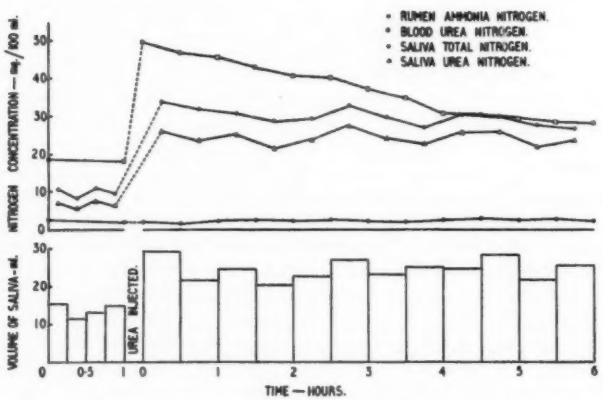

Fig. 1. The influence of an intracarotid infusion of urea on the concentration of blood urea nitrogen, rumen ammonia nitrogen, and the total and urea nitrogen in the parotid saliva secreted by a single gland in a sheep.

TABLE 1.

The secretion of salivary nitrogen by both parotid glands in a sheep before and after the intravenous injection of urea.

Time (hours)	Volume of saliva (ml.)		Concentration of total nitrogen (mg. per 100 ml.)		Concentration of urea nitrogen (mg. per 100 ml.)		Blood urea nitrogen (mg. per 100 ml.)	Rumen ammonia (mg. per 100 ml.)
	L. gland	R. gland	L. gland	R. gland	L. gland	R. gland		
0—¼	12·2	11·1	8·2	7·8	5·9	5·5	22·2	2·7
¼—½	13·4	12·6	8·8	8·0	6·1	5·9		
½—¾	14·7	13·5	9·1	8·7	6·7	6·2		
¾—1	12·9	13·3	7·9	8·4	5·6	5·8	21·7	2·2
1—¼	1·4 g. Urea nitrogen given by intravenous injection							
0—½	26·8	24·2	29·4	27·8	25·3	23·6	42·3	2·0
½—1	22·4	25·4	26·1	30·3	22·4	24·7	46·1	2·4
1—1½	27·2	29·4	26·9	32·4	23·8	27·1	38·7	2·1
1½—2	23·0	23·9	27·8	25·8	22·7	21·0	35·8	2·6
2—2½	19·8	22·5	23·6	24·9	21·1	22·3	36·4	3·0
2½—3	26·0	20·9	30·2	23·6	25·1	19·3	33·8	3·4
3—3½	22·4	24·3	24·4	26·2	22·6	23·1	29·9	4·0
3½—4	26·2	22·9	28·2	21·5	23·4	19·7	30·7	3·2
4—4½	23·7	24·8	23·7	25·3	21·8	20·3	27·6	2·8
4½—5	25·6	27·3	29·8	26·7	22·9	22·1	25·8	2·6
5—5½	20·6	25·8	23·1	24·6	19·4	21·5	24·7	2·1
5½—6	23·5	29·6	25·6	27·2	20·9	23·7	26·1	2·5

Because of the responses which might have been predicted to occur from the results obtained using animals with permanent unilateral parotid fistulae, identical experiments were carried out on a sheep with both parotid ducts cannulated. The results of such an experiment have been presented in Table 1 and Fig. 2.

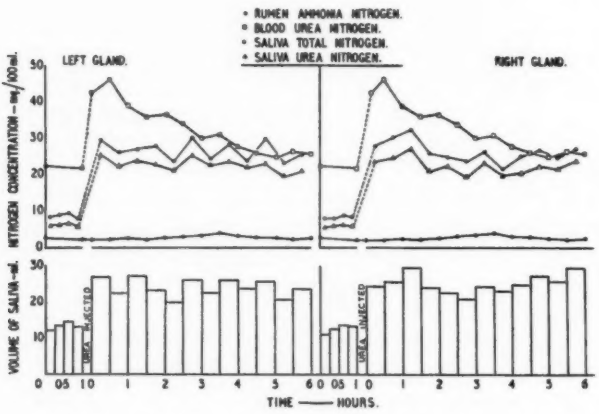

Fig. 2. The influence of an intracarotid infusion of urea on the concentration of blood urea nitrogen, rumen ammonia nitrogen, and the total and urea nitrogen in the parotid saliva secreted by the left and right gland of a sheep.

In each instance the blood urea nitrogen concentration was increased immediately following the urea infusion to approximately 2 to 2½ times that during the control period. The concentration of blood urea nitrogen then steadily declined over the remaining six hours but had still not returned to the pre-injection level at that time. These results indicate, nevertheless, that a considerable amount of the injected urea had been cleared from the blood.

As was anticipated from the report of Simonnet *et al.* (1957), part of this urea clearance occurred via the parotid secretions. In the half-hour samples taken immediately following the injection of urea, the concentration of urea nitrogen in the saliva increased by as much as 4 to 6 times that of the control period. More important, however, was the fact that the concentration of urea nitrogen in the saliva continued to remain higher than the pre-injection level for the next 6 hours and that it did not completely parallel the changes that occurred in the blood. Although Somers (1961a) found that the concentration of urea nitrogen in the saliva of sheep could be related to the rate of secretion, the observed increases in the results under discussion did not coincide with any marked changes in the rate of secretion (see Fig. 2).

TABLE 2.

The volume of parotid saliva secreted half-hourly by each gland in the one sheep and the amount of total nitrogen and urea nitrogen in the saliva before and after the intravenous injection of urea.

		Half hourly time intervals													
		Pre-urea injection		Post-urea injection											
		1	2	1	2	3	4	5	6	7	8	9	10	11	12
Volume of saliva (ml.)	L.G.	25·6	27·6	26·8	22·4	27·2	23·0	19·8	26·0	22·4	26·2	23·7	25·6	20·6	23·5
	R.G.	23·7	26·8	24·2	25·4	29·4	23·9	22·5	20·9	24·3	22·9	24·8	27·3	25·8	29·6
Total nitrogen (mg.)	L.G.	2·2	2·4	7·9	5·6	7·3	6·4	4·7	7·9	5·5	7·4	5·6	7·6	4·8	6·0
	R.G.	1·9	2·3	6·7	7·7	9·5	6·2	5·6	4·9	6·4	4·9	6·3	7·3	6·4	8·1
Urea nitrogen (mg.)	L.G.	1·5	1·7	6·8	5·0	6·5	5·2	4·2	6·5	5·1	6·1	5·2	5·9	4·0	4·9
	R.G.	1·4	1·6	5·7	6·3	8·0	5·0	5·0	4·3	5·6	4·5	5·0	6·0	5·6	7·0
Ratio of urea nitrogen : total nitrogen (p.c.)	L.G.	68·2	70·8	86·1	89·2	89·0	81·3	89·4	82·3	92·7	82·4	92·9	77·6	83·3	81·7
	R.G.	73·7	69·6	85·1	84·2	84·2	80·6	89·3	87·8	87·5	91·8	79·4	82·2	87·5	86·4

Note: L.G. and R.G. refer to the left and right parotid glands respectively.

The data in Table 1 have been used to prepare Table 2 to show the volume of saliva secreted by each gland over the half-hourly periods and the actual amount of total nitrogen and urea nitrogen in the saliva before and after the injection of urea.

Apart from demonstrating the very close similarity between the secretory response of the left and right gland in the one sheep the data in Table 2 also show the sustained increase in the amount of total nitrogen and urea nitrogen in the saliva after the injection of urea, notwithstanding the simultaneously declining blood urea nitrogen concentration, and the small amount of variability in the rate of secretion.

One other feature arising from these results is the higher ratio of urea nitrogen : total nitrogen in the saliva in the post-injection period compared with the ratio in the pre-injection samples. Examination of the raw data from other experiments showed the consistency of this trend, the magnitude of the increase being of the order of 10 p.c. (Table 2). However, despite the higher percentage of the total nitrogen accounted for by the urea nitrogen, the amount of nitrogen, other than urea nitrogen, also increased in the post-injection samples of saliva. No satisfactory explanation could be found in the experiments to explain this phenomenon.

From a consideration of these combined observations it appeared that the secretion of urea in the parotid saliva is actively regulated by mechanisms within the glands themselves.

The influence of injected urea on the daily salivary nitrogen secretion and on the overall nitrogen metabolism of the sheep.

A complete summary of the means and the daily range for each component at the two levels of nitrogen intake, with and without the daily addition of 1·4 gm. of urea nitrogen to the blood, has been presented in Table 3 for the three sheep.

The results in Table 3 call for some comment. In the first instance, although the three sheep showed unequivocal treatment responses, the magnitude of the response was not exactly the same in each animal. However, because the treatment responses were so clearly defined in the mean values for each animal, the subsequent discussion and conclusions have been made on group means.

It can be seen from Table 3 that, as the nitrogen intake fell from 14·5 to 6·8 gm. per day, the amount of total nitrogen in the saliva also fell from 294 to 117 mg. per day, while the amount of urea nitrogen fell from 198 to 78 mg. per day. At both the high and low levels of nitrogen intake the urea nitrogen accounted for some 67 p.c. of the total salivary nitrogen. The smaller quantities of total nitrogen and urea nitrogen secreted in the saliva when the diet containing the low level of nitrogen was fed, were not due to a reduced daily flow of saliva. In fact, a larger volume of saliva was produced daily on this ration, which was no doubt due to the higher proportion of fibre it contained. Ellen-

TABLE 3.

Summary of main experimental results from daily collections.

	Sheep no.	High nitrogen intake		Low nitrogen intake	
		Without blood urea injections	With blood urea injections	Without blood urea injections	With blood urea injections
Daily volume of saliva (ml.)	1	2355 (1975-2633)	2486 (2165-2831)	3207 (2768-3718)	3363 (2947-3819)
	2	2763 (2430-3214)	2805 (2493-3341)	3412 (2957-3658)	3272 (2795-3641)
	3	2654 (2315-3018)	2578 (2294-3115)	3342 (3009-3710)	3473 (3200-3810)
Group mean		2591	2623	3320	3369
Total nitrogen in saliva mg. per day)	1	286 (251-327)	449 (410-497)	111 (87-130)	461 (394-527)
	2	307 (254-372)	435 (362-481)	124 (106-152)	474 (395-533)
	3	290 (239-344)	460 (413-508)	115 (91-135)	502 (427-561)
Group mean		294	448	117	479
Urea nitrogen in saliva (mg. per day)	1	190 (163-229)	331 (289-376)	74 (61-87)	377 (289-458)
	2	209 (176-257)	318 (266-374)	84 (70-102)	378 (301-432)
	3	196 (158-241)	310 (247-388)	77 (62-93)	409 (336-476)
Group mean		198	320	78	388
Total nitrogen in urine (gm. per day)	1	6·65 (6·12-7·33)	7·54 (7·01-8·29)	3·66 (3·38-4·17)	3·94 (3·76-4·27)
	2	7·30 (6·65-7·91)	8·23 (7·63-8·81)	3·58 (3·16-4·12)	3·88 (3·41-4·31)
	3	6·66 (6·09-7·13)	7·66 (7·12-8·09)	3·88 (3·54-4·16)	4·18 (3·71-4·53)
Group mean		6·87	7·81	3·71	4·03
Urea nitrogen in urine (gm. per day)	1	5·42 (4·95-6·03)	6·39 (5·88-7·02)	2·48 (2·19-2·85)	2·77 (2·35-3·36)
	2	5·97 (5·37-6·54)	6·96 (6·33-7·64)	2·41 (1·95-2·87)	2·79 (2·23-3·25)
	3	5·47 (4·87-5·89)	6·48 (5·48-6·98)	2·72 (2·20-3·13)	2·93 (2·46-3·31)
Group mean		5·62	6·61	2·54	2·83
Total nitrogen in faeces (8 day period) (gm. per day)	1	4·67	4·73	3·35	3·65
	2	4·31	4·25	3·47	3·29
	3	4·82	4·95	3·68	3·88
Group mean		4·45	4·64	3·50	3·61
Average nitrogen balance (gm. per day)	1	+3·21	+3·66	−0·25	+0·57
	2	+2·92	+3·45	−0·29	+0·99
	3	+3·05	+3·32	−0·80	+0·10
Group mean		+3·06	+3·48	−0·45	+0·55

berger and Hofmeister (1887), Block (1939) and Denton (1956) have observed that changes in the physical composition of the diet have a significant influence on the amount of saliva secreted.

Reference to Table 3 shows that the injection of urea nitrogen into the blood of the sheep at the rate of 1·4 gm. of urea nitrogen per day when the animals were on a high nitrogen intake caused the total salivary nitrogen to increase by approximately 150 mg. per day. In this case there was little change in the ratio of total nitrogen : urea nitrogen in the total daily saliva compared with the ratio during the normal feeding period. At the low level of nitrogen intake the injection of 1·4 gm. of urea nitrogen into the blood daily resulted in an increase of approximately 350 mg. per day in the amount of total salivary nitrogen, and a corresponding increase of some 300 mg. per day in the salivary urea nitrogen. There was, however, a disproportionate increase in the output of urea nitrogen in the saliva over the whole period during which the urea injections were being made compared with the composition of the saliva secreted under normal feeding conditions.

A similar increase was also observed in the short-term experiments reported in the previous section, where urea was injected into the blood after the animals had been fasted for 24 hours. Seemingly the immediate physiological condition of the fasted animals was analogous to that of the animals over the whole period of low nitrogen intake, and therefore the injection of urea nitrogen into the blood elicited the same response in each instance.

Although it is known that the concentration of blood urea nitrogen is influenced by the rumen ammonia levels (Lewis, 1957) it could be anticipated that only low levels of rumen ammonia would be produced from the diet containing the low level of nitrogen, and therefore that the diurnal variations in the blood urea nitrogen concentration would be small. Similarly, little change in the blood urea nitrogen concentration could be expected from the production of ammonia in the rumen after the animals had been denied food for 24 hours.

Apart from the increases which occurred in the salivary nitrogen secretion from adding 1·4 gm. of urea nitrogen to the blood daily, significant changes also took place in the excretion of urinary nitrogen. When the nitrogen input was elevated from 14·5 gm. per day to 15·9 gm. per day by the addition of the 1·4 gm. of urea nitrogen to the blood, the excretion of urinary nitrogen increased by approximately 1·0 gm. per day and this was due almost entirely to an increase in the urinary excretion of urea nitrogen. However, even at this level of nitrogen input there was an apparent retention of 0·4 gm. of the injected urea nitrogen daily, and of this nitrogen approximately 0·1 gm. could be accounted for in the saliva secreted from one parotid gland.

When the nitrogen input of the animals on the diet low in nitrogen was increased from 6·8 to 8·2 gm. per day by the daily injection of 1·4 gm. of urea nitrogen into the blood, in addition to a fourfold increase in the salivary total nitrogen and slightly greater increase in the salivary urea nitrogen, there

was also a higher urinary nitrogen excretion. In this case, however, the urinary nitrogen excretion was only increased by approximately 0·3 gm. per day, and again this increase could be almost entirely accounted for by the increased amount of urea nitrogen in the urine. Most of the remainder of the 1·4 gm. of urea nitrogen injected daily was retained by the sheep, the mean nitrogen balance increasing from −0·45 gm. per day to +0·55 gm. per day. In this instance, each parotid gland returned approximately 0·3 gm. of the retained urea nitrogen to the rumen daily.

DISCUSSION.

The results of both the short-term experiments and those made on a daily basis clearly demonstrate that increasing the level of blood urea by directly injecting urea into the blood resulted in the secretion of a larger amount of urea nitrogen and total nitrogen in the parotid saliva of sheep. However, the responses in the salivary secretions were not standard when an equivalent amount of urea nitrogen was injected into the blood of animals under different physiological conditions. Where urea injections were made into the blood of animals fasted for 24 hours, or of those on a low nitrogen intake, not only was there an increase in the amount of total nitrogen and urea nitrogen in the saliva, but also a higher proportion of the total occurred as urea. The higher ratio of urea nitrogen : total nitrogen observed to follow the urea injections suggests that mechanism(s) other than passive diffusion operate in the transfer of urea from the blood into the saliva. It can be seen from Figs. 1 and 2, however, that the concentration of salivary urea nitrogen at no time exceeded that in the blood, thus the nature of the mechanism(s) did not appear to be one responsible for actively concentrating the blood urea.

The injection of the same amount of urea into the animals when on a high plane of nitrogen nutrition did not create a proportional response compared with the two categories previously mentioned. Nor did the injection of urea into these animals significantly alter the ratio of urea nitrogen : total nitrogen in the saliva.

These observations indicate that it is possible to saturate the transport mechanism(s) and that there is a limit value imposed on the secretion of total nitrogen and urea nitrogen by the glands. The results of the short-term experiments suggest that the limit value is approximately 30 mg. per 100 ml. in the case of the total salivary nitrogen and 25 mg. per 100 ml. for the salivary urea nitrogen. This limit value for the salivary urea nitrogen appeared to be held at all blood urea nitrogen concentrations down to this value at least.

While Schmidt-Nielsen *et al.* (1957) found that almost all of the urea injected into the camel on a low nitrogen intake was retained, the output of urinary nitrogen in the sheep under discussion was a reflection of both the total nitrogen intake and the urea nitrogen injected into the blood. Nevertheless, under conditions where an economy of nitrogen was important to the sheep,

much less of the injected urea was excreted in the urine than when the same animals received urea injections in addition to a diet which provided ample nitrogen.

In so far as the lack of nitrogen resulted in an increased retention of the injected urea nitrogen in the region of negative nitrogen balance, at least the return of nitrogen, and particularly urea nitrogen to the rumen via the parotid saliva, did provide a substantial part of an overall mechanism for the useful retention of the added nitrogen. However, even under these conditions it is clear that the proportion of the total retained urea nitrogen returned to the rumen in the parotid secretions did not exceed 50 p.c. of the total urea nitrogen injected into the blood.

The high proportion of the retained urea nitrogen which did appear in the parotid saliva, however, became a significant part of the total 1·4 gm. of urea nitrogen injected into the sheep daily.

Although the other salivary secretions of sheep have not been analysed for urea they do contain nitrogen and it seems probable that they too may contribute to the useful retention of the injected urea where a conservation of nitrogen is important to the sheep. While the diffusion of urea into the rumen through the epithelial lining which has been shown to occur in sheep (Simonnet et al., 1957) no doubt also contributes to the overall mechanism of urea retention, the present investigations show that the parotid secretions are a significant factor in the nitrogen economy of the ruminant at low levels of nitrogen intake.

REFERENCES.

Block, E. L. (1939): Schenov. J. Physiol. U.S.S.R., 27, p. 200.
Colin, G. C. (1886): Traité de physiol. comparée des animaux. Vol. 1, 3rd. ed., Paris.
Conway, E. J. (1950): Microdiffusion Analysis and Volumetric Error. 3rd. ed. Crosby, Lockwood and Sons, London.
Denton, D. A. (1956): J. Physiol., 131, p. 516.
Denton, D. A. (1957): Quart. J. exp. Physiol., 42, p. 72.
Ellenberger, W., and Hofmeister, V. (1887): Arch. Anat. Physiol. Lpz. (Suppl.), 138, p. 147.
Jarrett, I. G. (1948): J. C.S.I.R., 21, p. 311.
Lewis, D. (1957): J. agr. Sci., 48, p. 438.
McDonald, I. (1948): Thesis, University of Cambridge.
McKenzie, H. A., and Wallace, H. S. (1954): Aust. J. Chem., 7, p. 55.
Moir, R. J., and Somers, M. (1957): Aust. J. agr. Res., 8, p. 253.
Moir, R. J., and Williams, V. J. (1950): Aust. J. sci. Res., 3, p. 381.
Somers, M. (1957): Aust. vet. J., 33, p. 297.
Somers, M. (1958): Nature, 182, p. 400.
Somers, M. (1961a): Aust. J. exp. Biol., 39, p. 111.
Somers, M. (1961b): *Ibid.*, 39, p. 123.
Somers, M. (1961c): *Ibid.*, 39, p. 133.
Schmidt-Nielsen, B., Schmidt-Nielsen, K., Houpt, T. R. and Jarnum, S. A. (1957): Am. J. Physiol., 188, p. 477.
Simonnet, H., le Bars, H., and Molle, J. (1957): Com. rendus Acad. Sci., Paris, 244, p. 943.
Scheunert, A., and Krzywanek, Fr. W. (1929): Pflug. Arch. ges. Physiol., 223, p. 472.
Willits, C. O., Coe, M. R., and Ogg, C. L. (1949): J. Ass. Off. Agric. Chem., 32, p. 118.
Wu, D. Y., and Wu, H. (1951): Proc. Soc. exp. Biol. Med., 76, p. 130.

PHAGOCYTOSIS BY EOSINOPHILS FOLLOWING ANTIGEN-ANTIBODY REACTIONS *IN VITRO*

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SUMMARY.

Phagocytosis by human and rat eosinophils was studied by means of an *in vitro* system consisting of red cells as antigen, immune serum and white cells. Erythrophagocytosis by the white cells, including eosinophils, occurred following the reaction between red cell antigens and fresh immune sera at room temperature. A high percentage of the eosinophils showed phagocytosis. The part played by complement in phagocytosis was investigated by means of immune serum inactivated by heat, hydrazine, zymosan and diethyl ether. It was concluded that the first component is the only component of complement required for phagocytosis.

INTRODUCTION.

The function of the eosinophils remains obscure. At one time these cells were thought to contain histamine but it is now known that the bulk of the histamine in the blood resides in the basophils (Graham, Lowry, Wheelwright, Levy and Parish, 1955). Vercauteren and Peeters (1952) showed that an extract of eosinophil granules decreased the contraction of smooth muscle caused by histamine and suggested that the eosinophil granules had antihistamine properties. The preparations of eosinophil granules used were by no means pure and the effect on smooth muscle was so slight as to be of doubtful physiological significance. It has been shown by Archer (1960) that aqueous extracts of eosinophils will decrease by 60 p.c. the oedema produced in the skin of horses following the injection of histamine but this author states that the eosinophil probably has functions other than the direct inactivation of histamine. Eosinophils are known to accumulate in immunised animals at the site of injection of antigen (Speirs, 1958; Litt, 1960; Bosworth and Archer, 1961) and this finding led Speirs to the belief that the eosinophils take up antigen and transport it in modified form to the reticulo-endothelial system.

It is known that eosinophils are phagocytic cells (Mesnil, 1895; quoted by Vaughan, 1953) and in the present work phagocytosis by rat and human eosinophils was studied following antigen-antibody reactions *in vitro*.

MATERIALS AND METHODS.

Rat white cells including eosinophils were obtained from the peritoneal cavity of rats by a method previously described (Archer, 1959). They were mainly mononuclear cells and eosinophils. Neutrophils were absent. The peritoneal cavity of each rat was washed with physiological saline twice weekly for several weeks prior to the collection of the cells. This treatment has been shown to increase the yield of eosinophils from the peritoneal cavity and to decrease the number of mast cells present (Bosworth and Archer, 1961). After collection from the peritoneal cavity, a 2 p.c. suspension of cells was prepared in physiological saline.

Human white cells were obtained from bottles of group O blood by a method previously described (Archer and Kooptzoff, 1958). The final buffy coat preparation was washed three times in physiological saline and diluted to a 2 p.c. suspension in saline.

Rat immune sera. Human red cells were thrice washed in physiological saline and a 25 p.c. suspension of the cells was prepared in saline. 1 ml. of the suspension was injected into the thigh of the rat once a week for at least four weeks. Blood was obtained from the jugular vein from 5 to 10 days after the last injection. Four drops of serum when added to 1 drop of 25 p.c. human red cell suspension caused haemolysis within five minutes at room temperature. Normal control sera did not haemolyse human red cells at this temperature.

Human sera. Immune anti-A serum was obtained from group B blood donors. The sera used haemolysed group A red cells within five minutes at room temperature. Control sera were obtained from group A persons.

Inactivation of sera. Rat and human sera were inactivated as follows:

1. Heat: Fresh serum was heated at 56° C. for 30 minutes.

2. Hydrazine: Two drops of 0·1 M hydrazine solution at pH 7 were added to eight drops of serum and the mixture was incubated at 37° C. for 15 minutes. In some experiments the mixture was incubated at 37° C. for 60 minutes.

3. Diethyl ether: Method described by Klein (1960).

4. Zymosan: Three mg. zymosan obtained from Nutritional Biochemicals were added to each 1 ml. of fresh serum and the mixture was incubated at 37° C. for 30 minutes followed by centrifugation at 3,000 r.p.m. for 15 minutes to deposit the zymosan.

It is generally agreed that inactivation of complement by heat destroys the first component (C′1) and the second component (C′2) of complement. The third component (C′3) is destroyed following treatment with zymosan. Klein has shown that serum treated with ether or hydrazine results in a loss of the fourth component of complement (C′4). On prolonged incubation with hydrazine the serum was found also to lose C′1 activity.

Antigens for in vitro *test.* Thrice-washed human red cells were used in a strength of 25 p.c. in saline. Human red cells of any ABO group were used in experiments with rat immune sera. Group A red cells were used in experiments with anti-A sera.

In vitro *system.*

A mixture of three drops undiluted serum, one drop white cell suspension and one drop antigen solution was prepared in a test tube. Carbogen gas (O_2 90 p.c.; CO_2 10 p.c.) was blown over the surface of the mixture and the tube sealed with a rubber cork to maintain the pH at approximately 7. After five minutes at room temperature the tube and contents were centrifuged and the supernatant discarded. A film of the cell deposit was made on a glass slide. After staining with Leishman's stain the cells were examined under the microscope and those showing phagocytosis were counted.

In order to determine, if possible, which fraction of complement was necessary for phagocytosis to occur human red cells were in some experiments added in the cold to ether-

treated immune rat serum according to the method of Klein (1960). The cells and serum were held at 0° C. for 1 hour, after which time the sensitised cells were washed in citrate saline and taken up in barbitone buffer as described in this method to give an approximately 5 p.c. suspension. One drop of this sensitised red cell suspension was added to one drop of test solution and one drop of a suspension of rat white cells. The following test solutions were used: physiological saline, 10 p.c. bovine albumin in saline, 0·1 p.c. protamine sulphate in saline, normal rat serum and immune rat serum following inactivation by heat or ether. After five minutes at room temperature stained films were prepared and examined microscopically to determine the percentage of cells showing phagocytosis.

RESULTS.

Experiments with human red cells, rat white cells and rat immune sera.

Phagocytosis of human red cells by rat white cells occurred within five minutes of the addition of the cells to fresh rat immune serum held at room temperature and pH 7. The results of one experiment are shown in Table 1. It will be seen that 60 p.c. of the eosinophils had ingested red cells while only 8 p.c. of the mononuclear cells showed phagocytosis. Although the mononuclear cells outnumbered the eosinophils, the nett result was that more red cells had been ingested by the eosinophils than by the mononuclear cells. Microscopic examination of stained films revealed loss of eosinophil granules in those cells showing erythrophagocytosis. Coalescence of eosinophil granules was sometimes seen round the ingested red cell.

When normal rat serum or heat inactivated immune serum was substituted for fresh immune serum phagocytosis did not occur. Phagocytosis by eosinophils was reduced from 60 to 11 p.c. in preparations containing zymosan-inactivated immune serum. Results with hydrazine-inactivated serum were variable, depending upon the time of treatment. The number of eosinophils showing phagocytosis was high (54 p.c.) when immune serum was incubated with hydrazine for only 15 minutes before the addition of cells. Incubation with hydrazine for 60 minutes was found to decrease eosinophil phagocytosis to 5 p.c. Haemolysis of the added red cells occurred with fresh immune serum

TABLE 1.

Phagocytosis by rat peritoneal cavity cells of human red cells sensitised in rat immune serum.

Rat immune serum	Percentage phagocytosis	
	Eosinophils	Mononuclear cells
Fresh	60	8
Heat inactivated (56°C. for 30 mins.)	0	0
Zymosan inactivated	11	8
Hydrazine inactivated (37°C. for 15 mins.)	54	7
Normal rat serum control	0	0

but did not occur with the normal serum or with sera inactivated by heat, hydrazine or zymosan.

Experiment with human red cells sensitised in ether-treated immune rat serum.

Human red cells were added to ether-treated immune rat serum at 0° C. according to the method described by Klein (1960). Phagocytosis by rat white cells of the washed sensitised red cells occurred as shown in Table 2. In a

TABLE 2.

Phagocytosis by rat peritoneal cavity cells of human red cells sensitised in ether-treated immune serum (EAC'1 cells).

5 p.c. suspension of washed EAC'1 cells in barbitone buffer at pH 7·4 added to equal volumes of rat white cell suspension and of:	Percentage phagocytosis	
	Eosinophils	Mononuclear cells
Physiological saline	20	3
10 p.c. Bovine albumin in saline	43	10
0·1 p.c. Protamine sulphate in saline	30	11
Normal rat serum	50	7
Heat-inactivated immune serum	45	6
Ether-treated immune serum	44	8

saline suspension of sensitised red cells and rat white cells 20 p.c. of the latter showed phagocytosis. The percentage phagocytosis was approximately doubled when the cells were suspended in bovine albumin, protamine sulphate, normal rat serum, or immune rat serum inactivated by heat or by treatment with ether instead of in saline. Phagocytosis of the sensitised cells was probably increased non-specifically in the protein-containing media.

Experiments with group A red cells, group O white cells and immune anti-A serum.

In these experiments washed group A red cells were added to a mixture consisting of group O white cells and fresh serum from a group B donor containing anti-A haemolysins. Group O white cells were used instead of group A to avoid a reaction between the white cells and the immune serum.

Phagocytosis of group A red cells by the white cells occurred as shown in Table 3. It will be seen that 53 p.c. eosinophils, 47 p.c. neutrophils and 28 p.c. monocytes had ingested red cells. Phagocytosis did not occur when the immune anti-A serum was inactivated by heat or when the anti-A serum was replaced by anti-B serum. Following treatment of immune serum with zymosan, phagocytosis by eosinophils, neutrophils and monocytes was found in 39 p.c., 45 p.c. and 17 p.c. of cells respectively. Treatment of immune serum for 15 minutes with hydrazine reduced the percentage phagocytosis to 29 in

TABLE 3.

Phagocytosis by group O white cells of group A red cells sensitised in immune anti-A serum.

Serum	Percentage phagocytosis		
	Neutrophils	Eosinophils	Monocytes
Fresh immune anti-A	47	53	28
Heat-inactivated anti-A	0	0	0
Hydrazine-treated anti-A	33	29	14
Zymosan-treated anti-A	39	45	17
Anti-B serum control	0	0	0

eosinophils, 33 in neutrophils and 14 in monocytes. Haemolysis of added red cells occurred with fresh immune serum, but did not occur with the other sera.

Microscopic examination of stained films revealed loss of eosinophil granules in those eosinophils showing erythrophagocytosis. Large masses of eosinophilic material were sometimes seen round the ingested red cells.

DISCUSSION.

Eosinophils have been shown to accumulate at sites of antigen-antibody reaction (Speirs, 1958) and the cells are known to be phagocytic (Mesnil, 1895; quoted by Vaughan, 1953). The present experiments were undertaken in an attempt to define the conditions necessary for phagocytosis by the eosinophils of foreign antigens. Red cells were used as the source of antigen and ingestion of intact red cells was readily seen by microscopic examination of stained films (Fig. 1). Phagocytosis by eosinophils of sensitised red cells was a conspicuous feature in preparations containing fresh immune serum. On the other hand, phagocytosis was not observed when the immune serum was heated to 56° C. for 30 minutes. This inactivation is known to destroy the first and second components of complement.

Recently Klein (1960) described a method for the preparation of red cells coated with immune antibody and the first component of complement, using serum treated with ether. These red cells have been termed EAC'1 cells. In the present series of experiments it was found that eosinophils, neutrophils and mononuclear cells ingested the EAC'1 cells to the same degree as fully sensitised cells. EAC'1 cells may be prepared by using serum treated for a short time with hydrazine instead of ether. Hydrazine destroys the fourth component of complement but with prolonged action also destroys the first component of complement (Klein, 1960). It was found that cells sensitised in hydrazine-treated serum were ingested to a variable extent depending on the time of incubation of the serum with hydrazine. Treatment of rat serum with hydrazine for only 15 minutes prior to this addition of red cells resulted in phagocytosis to the same extent as with fresh immune serum but phagocytosis was greatly

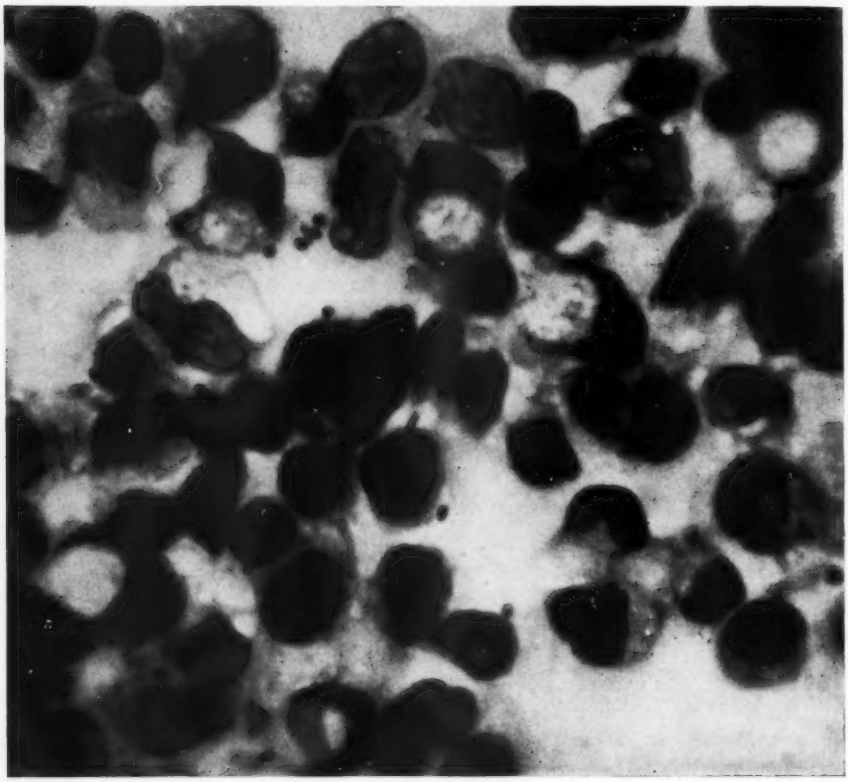

Fig. 1. A preparation of rat peritoneal cavity cells showing phagocytosis of sensitised human red cells by six eosinophils.

diminished by prolonged incubation of the serum with hydrazine. The findings are therefore consistent with the hypothesis that the first component is the only component of complement required for phagocytosis.

The experiments with human white cells showed that neutrophils and eosinophils readily ingested group A red cells suspended in immune anti-A serum. As the neutrophils outnumber the eosinophils in human blood by 15 to 20 times, it is obvious that under the experimental conditions neutrophils remove considerably more of the sensitised red cells. A feature noticed with human and rat eosinophils was the disappearance of granules from the cytoplasm around the ingested cell. The reason for this phenomenon is not clear but is being further investigated.

The rôle of the eosinophil in erythrophagocytosis has probably been overlooked in the past because the eosinophils comprise only a small percentage of the total leucocyte count in normal blood. It is not known whether their rôle is limited merely to the removal of antigenic material or whether the eosinophils play a special part in the immune reaction.

REFERENCES.

Archer, G. T. (1959): Austral. J. exp. Biol., 37, p. 383.
Archer, G. T., and Kooptzoff, Olga (1958): *Ibid.*, 36, p. 373.
Archer, R. K. (1960): Brit. J. Haemat., 6, p. 229.
Bosworth, Nola, and Archer, G. T. (1961): Austral. J. exp. Biol. (in press).
Graham, Helen T., Lowry, G. H., Wheelwright, Frances, Levy, Miriam A., and Parish, H. H. (1955): Blood, 10, p. 467.
Klein, P. G. (1960): J. exp. Med., 111, p. 77.
Litt, M. (1960): Blood, 16, p. 1318.
Speirs, R. S. (1958): Ann. N.Y. Ac. Sci., 73, pp. 283-306.
Vaughan, J. (1953): Blood, 8, p. 1.
Vercauteren, R., and Peeters, G. (1952): Arch. internat. Pharmacol., 89, p. 10.

THE EOSINOPHIL CONTENT OF THE PERITONEAL CAVITY OF THE RAT

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SUMMARY.

The eosinophil content of the peritoneal cavity was studied in normal rats and in rats which had received injections into the peritoneal cavity of a number of substances. A non-specific increase in eosinophils occurred following the injection of physiological saline, histamine, serotonin and phenergan. There was a specific increase in eosinophils following a challenge injection of human red cells into animals previously sensitised to this antigen. Large numbers of eosinophils, with greatly reduced numbers of mast cells, were obtained simply by repeatedly irrigating the peritoneal cavity with large volumes of saline.

INTRODUCTION.

When the peritoneal cavity of the rat is washed with physiological saline the aspirated fluid contains mononuclear cells, mast cells and cells with eosin-staining granules. Welsh and Geer (1959) studied the granules with the electron microscope and concluded that the granules were identical with those in eosinophils in the circulating blood. The present work is concerned with a study of the normal eosinophil population of the peritoneal cavity of the rat. Attempts were also made to increase the yield of eosinophils by injection of histamine, serotonin, phenergan and foreign red cells.

MATERIALS AND METHODS.

Peritoneal cavity cells were obtained from adult female albino rats by a method similar to that described by Archer (1959). Under ether anaesthesia, 50 ml. of physiological saline were injected into the peritoneal cavity. After rocking to disperse the saline, the fluid was drained from the peritoneal cavity by means of a 14 s.w.g. needle and the volume measured. The number of cells in a final volume of 1 ml. was counted by the method described by Dacie (1956) for the estimation of leucocytes in blood. In some experiments the mast cells were also counted in these preparations.

Total eosinophil counts were performed by the method described by Huntsman, Doggett and Holtkamp (1959). A small volume of concentrated cell suspension was mixed with an equal volume of a solution containing 10 p.c. bovine serum albumin in saline and a film was prepared on a glass slide. After staining with Leishman's stain, 500 cells were examined to obtain differential counts.

Repeated washing of peritoneal cavity was studied in six animals. 50 ml. of physiological saline were injected into the peritoneal cavity every third day on four occasions. The volume

of the cell suspension obtained was measured and cell counts were performed on each occasion.

Effect of phenergan, histamine and serotonin. Phenergan was obtained from May and Baker in sterile ampoules in a concentration of 25 mg./ml. and was diluted to a concentration of 1/1,000 in sterile physiological saline. The pH of the preparation was found to be approximately 7. Histamine was obtained from British Drug Houses Ltd. in a concentration of 1 mg./1 ml. and was diluted to a concentration of 1/100,000 in physiological saline. The pH of the preparation was adjusted to 7 using N/10 sodium hydroxide. Serotonin was obtained from the Abbott Laboratories, North Chicago, as powdered serotonin creatinine sulphate and was made up to a concentration of 1/100,000 in sterile physiological saline at pH 7. Each of these substances was given to six rats. The solutions were injected in 2 ml. amounts into the peritoneal cavity twice daily for three days and the peritoneal cells harvested 24 hours after the final injection. Six control animals were given similar intraperitoneal injections of 2 ml. physiological saline.

Immunisation of rats to human red cells. The method used was similar to that described by Speirs (1958). Human red cells were washed thrice in physiological saline and made up to a 25 p.c. suspension in saline. On four occasions each a week apart 1 ml. of the suspension was injected subcutaneously into the thigh of each of six rats. Seven days after the last injection the animals were given an intraperitoneal injection of 1 ml. of the red cell suspension. The peritoneal cavity cells were collected four days later. Six control animals were each given an intraperitoneal injection of 1 ml. of lysed red cell suspension four days before collection of the peritoneal cavity cells. The red cells were lysed by freezing and thawing.

RESULTS.

Cellular content of peritoneal cavity.

The volume of fluid obtained from the peritoneal cavity after injection of 50 ml. physiological saline varied between 36 ml. and 46 ml. It was presumed that the injected solution remaining in the peritoneal cavity contained cells in similar concentration to that in the fluid recovered. The total number of cells present in the cavity was calculated from the proportion of the administered fluid recovered and the concentration of cells therein. Six rats were examined and a mean total cell count of 12·69 million obtained. The mean eosinophil count was 2·91 million, representing 23 p.c. of the total cells.

TABLE 1.

Effect of repeated washing with physiological saline on the cell content (in millions) of the peritoneal cavity of the rat. The values in this and the following Tables are given as the mean ± standard error.

Number of peritoneal irrigations	Total number of cells	Mast cells	Eosinophils	Eosinophils p.c.
1	12·69±1·52	0·90±0·14	2·91±0·32	22·9
2	28·11±1·56	0·04±0·007	9·09±0·91	32·3
3	27·08±2·35	0·04±0·007	8·56±0·48	31·6
4	24·89±2·44	0·03±0·006	7·69±1·52	30·9

Cellular content with repeated washings.

The cells recovered from the peritoneal cavity when saline was injected every third day are shown in Table 1. Twice the total number of cells was found on the second and subsequent washings as on the first and there was a marked fall in the number of mast cells while the number of eosinophils increased approximately three-fold. There were no significant differences in cell contents of the second, third and fourth washings.

The effect of pharmacological agents on cell content.

The three substances used in this work, phenergan, histamine and serotonin, were chosen because of their relation to allergic phenomena in which eosinophils appear to play a part. The materials were injected intraperitoneally as described earlier and the peritoneal cavity irrigated 24 hours after the last injection. The cell content of such preparations is shown in Table 2. Injection of small quantities of saline doubled the number of cells which were harvested. The three pharmacological agents increased still further both the total cell number and the eosinophil number, but no significant differences were found between the three substances. The proportion of eosinophils in these experiments varied between 17·7 p.c. and 24·9 p.c.

TABLE 2.

Effect of pharmacological agents on the mean cell content (in millions) of the peritoneal cavity of the rat.

Number of animals	Substance	Total number of cells	Eosinophils	Eosinophils p.c.
10	Normal control	12·55±0·64	2·39±0·19	19·0
5	Physiological saline	26·81±1·58	6·3 ±0·62	23·5
6	Phenergan	46·56±2·14	8·63±1·35	18·5
6	Histamine	53·59±5·67	9·47±1·27	17·7
5	Serotonin	50·47±6·21	12·58±3·82	24·9

Effect of antigen-antibody reaction on cell content.

Two groups of rats were used in these experiments. The first was immunised to human red cells as described earlier and each animal received a challenge inoculum of 1 ml. of 25 p.c. human red cell suspension. The total number of cells harvested four days later was 26·56 million, of which 11·18 million (42·1 p.c.) were eosinophils (Table 3).

The second group of rats was not immunised but each was injected with 1 ml. of haemolysed 25 p.c. human red cell suspension to make this experiment comparable with the first in which lysis was produced by immune haemolysins almost immediately after the injection. The mean total cells recovered from the peritoneal cavity of the second group of rats was 24·75 million, approximately the same as in the first group but only 4·47 million eosinophils (18 p.c.) were

TABLE 3.

Mean cell content (in millions) of the peritoneal cavity of normal rats and of immunised rats following an intraperitoneal injection of human red cells.

Group	Total number of cells	Eosinophils	Eosinophils p.c.
Immunised rats (6 animals)	26·56±6·20	11·18±2·10	42·1
Normal rats (6 animals)	24·75±2·55	4·47±0·47	18·1

present, significantly less than in the first group. Both the total count and the eosinophil count in the second group were comparable with the counts obtained following injections of saline in the earlier experiments (Table 2).

DISCUSSION.

The work which has been described was initially undertaken to develop a method for obtaining eosinophils in large numbers and for separating them from other cells. The peritoneal cavity of the rat provides a useful source of eosinophils for *in vitro* studies: the yield is considerably higher than from mice (Speirs, 1955) and is comparable with that from the peritoneal cavity of guinea pigs (Litt, 1960). The total number of cells can be increased by repeated injection of saline or by injection of various drugs. Probably the simplest method of obtaining preparations of eosinophils is to use rats whose peritoneal cavities have been repeatedly irrigated with large volumes of saline as the mast cell content of such preparations is low and eosinophils, almost free of other cells, can be obtained by centrifugation in media of various specific gravities.

The increase of both the total yield and of the proportion of eosinophils following injection of various drugs appears to be a non-specific phenomenon as it occurred with drugs of quite different pharmacological activities. The explanation of the rise is not known but is possibly the result of peritoneal irritation.

The preliminary work with antigen-antibody reactions involving red cells suggests that following the immune reaction there may be a specific increase in the eosinophil content of the peritoneal cells. This is in agreement with the work of Speirs (1958) who found an accumulation of eosinophils between the second and tenth day after a challenge injection into sensitised mice. The antigens used by Speirs included extracts of parasites, ragweed pollen extracts, tetanus and diphtheria toxoids, heterologous erythrocytes, foreign serum or serum albumin. Similar findings have been reported recently by Litt (1960) who found an increase in eosinophils in the peritoneal cavity of guinea pigs following repeated weekly injections of horse serum, haemocyanin or human serum albumin. The response occurred in immunised animals within three days following the injection of antigen and ceased within 12 days.

REFERENCES.

Archer, G. T. (1959): Austral. J. exp. Biol., 37, p. 383.
Dacie, J. V. (1956): "Practical Haematology" (2nd ed.), pp. 37-46. Churchill, London.
Huntsman, D. B., Doggett, M. C., and Holtkamp, D. E. (1959): Amer. J. clin. Path., 31, p. 91.
Litt, M. (1960): Blood, 16, p. 1318.
Speirs, R. S. (1955): Ann. N.Y. Ac. Sc., 59, pp. 706-731.
Speirs, R. S. (1958): *Ibid.*, 73, pp. 283-306.
Welsh, R. A., and Geer, J. C. (1959): Amer. J. Path., 35, pp. 103-111.

THE EFFECT OF CHLORAMPHENICOL AND OXYTETRACYCLINE ON THE FORMATION OF INTERMEDIATES IN TRYPTOPHAN BIOSYNTHESIS

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(*Accepted for publication 6th February, 1961.*)

SUMMARY.

Previous experiments on the effects of chloramphenicol and the tetracyclines on the biosynthesis of tryptophan in *Escherichia coli* have been extended using a number of tryptophan auxotrophs. The formation of indole, anthranilic acid and 5-dehydroshikimic acid is inhibited by chloramphenicol and oxytetracycline. There is some variation among different strains with respect to the sensitivity of the aromatic pathway to the antibiotics.

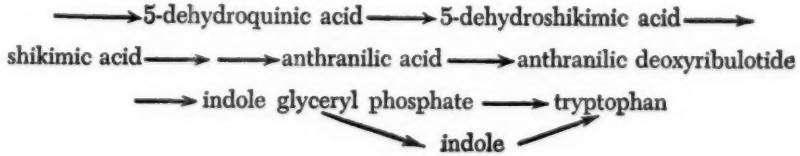
It is suggested that the antibiotics possibly interfere in some reaction leading to the formation of 5-dehydroshikimic acid. The present results provide further evidence that the mode of action of chloramphenicol and of the tetracyclines are related in some way.

INTRODUCTION.

Previous experiments (Gibson, McDougall, Jones and Teltscher, 1956b) have shown that bacteriostatic concentrations of chloramphenicol and the tetracyclines inhibit the biosynthesis of indole by cell suspensions of *Escherichia coli* 7-4. This tryptophan auxotroph was unable to convert indole to tryptophan and formed indole from glucose and ammonium ions, this formation being stimulated by the addition of serine.

The formation of other intermediates on the pathway of biosynthesis leading to tryptophan can be similarly studied using cell suspensions of auxotrophs blocked at other points (e.g. Gibson, Jones and Teltscher, 1956a; Doy and Gibson, 1959). Therefore by testing the action of the antibiotics on the formation of intermediates at various points in the biosynthetic sequence it might be possible to define their point, or points, of action.

Part of the generally accepted pathway of biosynthesis of tryptophan, in which all the intermediates are not yet known, is as follows:



The compounds which were chosen for study during the present work were indole, indole glyceryl phosphate (which is excreted from whole cells as indole glycerol), anthranilic acid and 5-dehydroshikimic acid.

MATERIALS AND METHODS.

Organisms used.

The strains of *E. coli* used were all mutants of *E. coli* 518 isolated after ultraviolet irradiation and penicillin selection using the technique described by Davis (1948). The strains required tryptophan or related compounds for growth or, in one case, a mixture of tryptophan, tyrosine and phenylalanine. The details of the growth requirements and compounds formed by cell suspensions are given in Table 1.

TABLE 1.

Strains of E. coli *used.*

Strain	Growth requirement*	Compound formed by cell suspensions
7-4	tryptophan	indole
R 2-13	tryptophan	indole
R 5-9	tryptophan	indole
R18-16	tryptophan	indole
7-8	indole or tryptophan	indole-3-glycerol
1-5	indole or tryptophan	anthranilic acid
7-2	shikimic acid or anthranilic acid or indole or tryptophan plus tyrosine and phenylalanine.	5-dehydroshikimic acid

* Supplement required for growth in glucose mineral salts medium. The concentration used was 10^{-4}M.

Media.

Heart infusion agar. This medium was used for the maintenance of stock cultures and for the growth of cells for suspensions. The preparation of this medium has been described previously (Gibson *et al.*, 1956a).

Synthetic medium. Bacteriostatic tests and tests for growth requirements were carried out using the chemically-defined medium described by Davis and Mingioli (1950). This medium was supplemented with amino acids as required. The medium was autoclaved at 10 lb./sq. in. for 10 min. when tubed.

Preparation and use of washed cell suspensions.

The preparation of suspensions has been described previously (McDougall and Gibson, 1959). Briefly, cells are harvested from overnight cultures on heart infusion agar plates, washed twice with saline and resuspended in phosphate buffer pH 7·7 to give a dry weight of cells of about 1·5 mg./ml.

The cell suspension was added to substrates to give a mixture with the following final concentrations:

glucose	0·1 *M*
NH_4Cl	0·005 *M*
$MgSO_4$	0·0001 *M*
phosphate buffer pH 7·7	0·1 *M*
cells	7·75 mg. dry wt./ml.

The mixture was incubated in a water bath at 37° C., sampled at intervals and the accumulated product estimated.

Bacteriostatic tests.

These have been described previously (McDougall and Gibson, 1958). Chloramphenicol and tetracycline solutions were pasteurised at 80° C. for 10 min. before use.

Estimation of indole.

The estimation of indole using Erhlich's reagent has been described previously (Gibson *et al.*, 1956a).

Estimation of anthranilic acid.

Two methods (colorimetric and spectrophotometric) were mainly used for the estimation of anthranilic acid. The results with the two methods agreed with one another and with those from a microbiological assay using a streptomycin resistant auxotroph responding to anthranilic acid.

Colorimetric method. This was a modification of the method described by Bratton and Marshall (1939) for the estimation of sulphonamides in blood and urine. The experimental sample was cooled, centrifuged and the clear supernatant decanted. The supernatant was then diluted (1/10 or 1/20) in distilled water to give a final volume of 10 ml.; 0·5 ml. of 4 *N* HCl was added and the tube cooled to about 2° C. by immersion in an ice bath. One ml. of 0·1 p.c. $NaNO_2$ was added, the tube shaken and allowed to stand for 3 min. in the ice bath after which 1 ml. of 0·5 p.c. ammonium sulphamate solution was added. The tube was shaken and allowed to stand for 2 min. in the ice bath. Then 1 ml. of 0·1 p.c. N-(1-naphthyl)-ethylenediamine hydrochloride was added, the tube shaken and placed in a 37° C. water bath for 1 hr. A reagent blank with distilled water in place of sample was prepared simultaneously. The intensity of the developed colour was estimated using an E.E.L. portable colorimeter with ½ in. tubes and filter no. 626 (maximum transmission 570 mμ.). The galvanometer reading was converted to concentration of anthranilic acid from a standard curve.

Spectrophotometric method. Anthranilic acid has an absorption spectrum in the ultraviolet region with two peaks (240 and 310 mμ.). Because of the greater non-specific absorption at 240 mμ. in experimental samples measurements of optical density at 310 mμ. were used to estimate anthranilic acid. The optical density of the supernatants from centrifuged experimental samples was measured in a Unicam spectrophotometer with 1 cm. cells using 0·1 *M* phosphate buffer (pH 7·7) as blank. A standard curve was prepared using synthetic anthranilic acid. When necessary, experimental samples were diluted using 0·1 *M* phosphate buffer.

Estimation of indole-3-glycerol.

This compound has an absorption spectrum similar to that of indole but the wavelength of maximum absorption is 278 mμ. The amount of indole-glycerol was estimated spectrophotometrically by measuring the optical density of experimental samples at 278 mμ. As a pure sample of indole-3-glycerol was not available to convert optical density readings to concentration of compound the results are reported as optical density at 278 mμ.

Estimation of 5-dehydroshikimic acid.

This compound has an absorption maximum at 234 mμ. The optical density at 234 mμ. was therefore used to estimate 5-dehydroshikimic acid. Estimations of concentration could be made from a standard curve constructed using a sample of pure 5-dehydroshikimic acid. However, in experimental samples there is some non-specific absorption at 234 mμ. so that the results are expressed as optical density at this wavelength.

RESULTS.

Examination of the rate of indole formation by *E. coli* 7-4 suggests an adaptive process. To eliminate the possibility that the observed results were merely a consequence of inhibition of adaptive enzyme formation maximum rate of synthesis was achieved before adding the antibiotic (Gibson *et al.*, 1956b). Streptomycin (150 μg./ml.) was often included among the additions to check that inhibition by the other antibiotics was not due to inhibition of an adaptive process (Gibson, *et al.*, 1956b).

Antibiotics and anthranilic acid formation by E. coli 1-5.

Both chloramphenicol (5 μg./ml.) and oxytetracycline (1 μg./ml.) markedly inhibited the formation of anthranilic acid from glucose and ammonium ions by *E. coli* 1-5 (Figs. 1 and 2). Under similar conditions streptomycin (150 μg./ml.) had no significant effect on anthranilic acid synthesis.

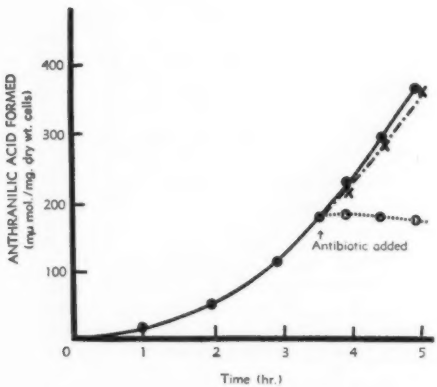


Fig. 1. Effect of chloramphenicol and streptomycin on synthesis of anthranilic acid by *E. coli* 1-5. The cells were incubated with substrates for 3½ hrs. at 37° C. before antibiotics were added. Anthranilic acid was estimated colorimetrically.

——— No antibiotic added.
---------- 5 μg./ml. chloramphenicol.
—·—·—·— 150 μg./ml. streptomycin.

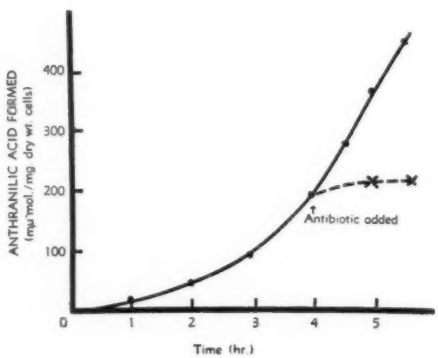


Fig. 2. Effect of oxytetracycline on anthranilic acid synthesis by *E. coli* 1-5. The cells were incubated at 37° C. for 4 hrs. before addition of the antibiotics. Anthranilic acid was assayed spectrophotometrically.

——— No antibiotic added.
– – – – – 1 μg./ml. oxytetracycline.

Antibiotics and indole-glycerol formation by E. coli 7-8.

At the above concentrations the antibiotics had relatively little effect on indole-glycerol synthesis by *E. coli* 7-8 (Fig. 3). Both chloramphenicol and the oxytetracycline had a greater inhibiting effect than streptomycin.

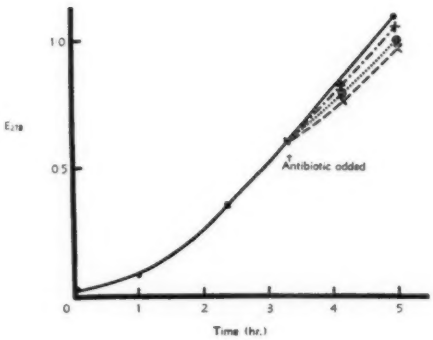


Fig. 3. Effect of antibiotics on synthesis of indole-3-glycerol by *E. coli* 7-8. Antibiotics added after adaptation period of 3 hrs.

——— No antibiotic added.
---------- 5 μg./ml. chloramphenicol.
– – – – – – 1 μg./ml. oxytetracycline.
—·—·—·— 150 μg./ml. streptomycin.

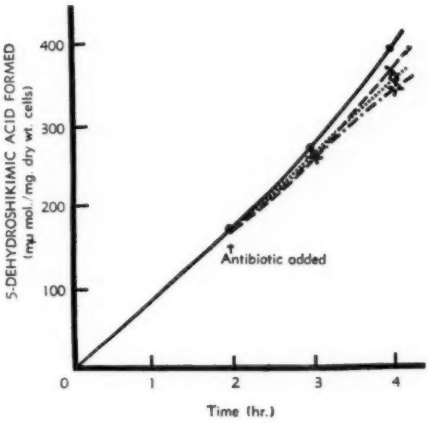


Fig. 4. Effect of antibiotics on synthesis of 5-dehydroshikimic acid by *E. coli* 7-2. Antibiotics added after adaptation period of 2 hrs.

——— No antibiotic added.
---------- 5 μg./ml. chloramphenicol.
– – – – – – 1 μg./ml. oxytetracycline
—·—·—·— 150 μg./ml. streptomycin.

Antibiotics and 5-dehydroshikimic acid formation by E. coli 7-2.

As with the formation of indole-glycerol, chloramphenicol (5 μg./ml.) or oxytetracycline (1 μg./ml.) had little effect on the formation of 5-dehydroshikimic acid by *E. coli* 7-2 (Fig. 4).

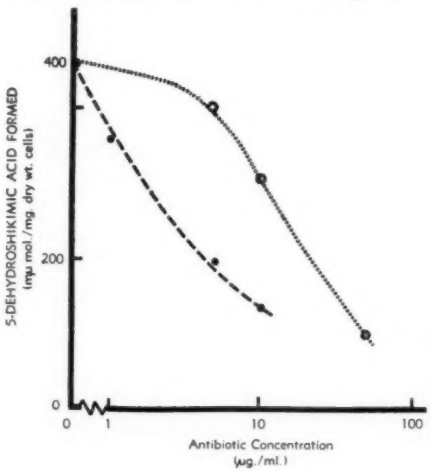


Fig. 5. Effect of increasing concentrations of chloramphenicol and oxytetracycline on the formation of 5-dehydroshikimic acid by *E. coli* 7-2. The results show the amount of 5-dehydroshikimic acid formed over a 2 hr. period in the presence of the amount of antibiotic shown. Cells were adapted for 2 hrs. before addition of antibiotics.

---------- Chloramphenicol.
– – – – – – Oxytetracycline.

When the effect of increasing concentrations of these two drugs were tried, however, it was found that they increasingly inhibited 5-dehydroshikimic acid synthesis (Fig. 5).

Antibiotics and indole formation by various strains of E. coli.

Because of the sensitivity to antibiotics of indole and anthranilic acid syntheses by *E. coli* 7-4 and *E. coli* 1-5 respectively and the relative insensitivity to antibiotics of indole-glycerol and 5-dehydroshikimic acid formation in the latter it was thought that this variation might be characteristic of the strain rather than an indication of which reactions were inhibited. Several auxotrophs of *E. coli* (all derived from *E. coli* 518) which accumulated indole were compared with respect to the effect of chloramphenicol and oxytetracycline on indole synthesis. The results are presented in Table 2 and it can be seen that there is considerable variation in sensitivity to the antibiotics, indole synthesis by *E. coli* 7-4 being much more sensitive than that by the other strains tested.

TABLE 2.

Effect of antibiotics on indole synthesis by various strains of E. coli.

	p.c. inhibition* by			
	chloramphenicol (μg/ml.)		oxytetracycline (μg/ml.)	
Strain	5	50	1	10
7-4	71	89	42	72
R 2-13	28	63	16	52
R 5-9	23	47	4	30
R18-16	29	59	0	27

* p.c. inhibition of indole synthesis during 2 hr. period following adaptation without antibiotics.

Sensitivity of various strains to growth inhibition by antibiotics.

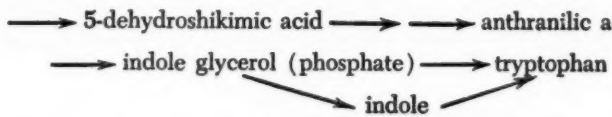
Despite the variable degree of inhibition in various strains of the synthesis of intermediates by antibiotics the growth of all strains is inhibited equally by the drugs. Thus 2 μg./ml. of chloramphenicol allowed growth while 5 μg./ml. inhibited in all cases. Similarly 0·5 μg. of oxytetracycline allowed growth in all cases while 1 μg./ml. was completely inhibitory.

DISCUSSION.

Experiments on the effects of antibiotics on the synthesis of aromatic compounds were previously carried out with *E. coli* 7-4, an auxotroph which formed indole from glucose and ammonium chloride (Gibson *et al.*, 1956b). It was

found then that indole formation was inhibited by very low concentrations of chloramphenicol and the tetracyclines.

The results presented above show that it was not possible, by simply testing the effects of low concentrations of the antibiotics on the formation of various intermediates in aromatic synthesis, to ascertain the point of action of the drugs. The pathway of synthesis may be represented briefly as follows:



Thus it was found that indole and anthranilic acid formation was inhibited by low concentrations of antibiotics but indole-glycerol and 5-dehydroshikimic acid synthesis only by rather higher concentrations.

It seemed therefore that the variation in sensitivity might be due to variation among strains of organisms used, even though they all came from the same parent strain, and a similar variation would be found among different strains forming the same intermediate. This assumption was supported by the results presented in Table 2 where the effects of different concentrations of antibiotics on several different strains forming indole are compared. In bacteriostatic tests all strains have the same sensitivity to each antibiotic.

Such variability among strains makes it difficult to define, using cell suspensions, the point of action of the antibiotics in their interference with tryptophan synthesis. The inhibition of 5-dehydroshikimic acid formation by 5 to 10 μg./ml. of oxytetracycline and 10 to 50 μg./ml. of chloramphenicol can probably still be considered significant. Such being the case, the point of action of the antibiotics on aromatic biosynthesis can be considered at some stage or stages before 5-dehydroshikimic acid. Such a conclusion is supported by the brief communication by Wolfe and Hahn (1960). They found that when cell suspensions of the auxotroph accumulating 5-dehydroshikimic acid were incubated under similar conditions to those described above fluorescent substances accumulated if chloramphenicol was present. Similar results were obtained with strains which accumulated indole or anthranilic acid.

If a specific reaction in aromatic biosynthesis is inhibited by the antibiotics then the point of action is most likely to be clearly resolved using cell free extracts. Some preliminary investigations have been carried out along these lines. Smith and Yanofsky (personal communication) have examined the effects of chloramphenicol and the tetracyclines on the conversion of anthranilic acid to indole by extracts of auxotrophs of *E. coli* K12. No significant inhibition was found. *Escherichia coli* 7-4 is an unusual strain of *E. coli* in that the effect of the mutation was to alter the B protein of tryptophan synthetase so that serine and pyridoxal phosphate stimulate the conversion of indole glyceryl phosphate to indole (Gibson, Gibson and Yanofsky, 1961). However, chloramphenicol

has no appreciable inhibitory effect on this conversion (Gibson, unpublished results).

The similarity of the effects of chloramphenicol and the tetracycline as shown in these experiments is in accord with the previous observations on effects on indole formation by both drug sensitive and drug resistant cells (Gibson *et al.*, 1956b). The observations of development of cross resistance between chloramphenicol and tetracycline resistant strains (Herrel, Heilman and Wellman, 1950; Pansy, Khan, Pagano and Donovick, 1950) provides further evidence that the mode of action of these antibiotics is related in some way.

There is no clear-cut evidence to suggest that the primary anti-bacterial effect of chloramphenicol and the tetracyclines is concerned with inhibition of aromatic synthesis. Nevertheless, the study of the action of these antibiotics on synthesis of aromatic compounds provides an experimental approach to the difficult problems of the mode of action of these drugs.

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REFERENCES.

Bratton, A. C., and Marshall, E. K. (1939): J. biol. Chem., 128, p. 537.
Davis, B. D. (1948): J. Am. chem. Soc., 70, p. 4267.
Davis, B. D., and Mingioli, E. S. (1950): J. Bact., 60, p. 17.
Doy, C. H., and Gibson, F. (1959): Biochem. J., 72, p. 586.
Gibson, F., Gibson, M. I., and Yanofsky, C. (1961): J. gen. Microbiol. (paper in the press).
Gibson, F., Jones, M. J., and Teltscher, H. (1956a): Biochem. J., 64, p. 132.
Gibson, F., McDougall, B., Jones, M. J., and Teltscher, H. (1956b): J. gen. Microbiol., 15, p. 446.
Herrel, W. E., Heilman, F. R., and Wellman, W. E. (1950): Ann. N.Y. Acad. Sci., 53, p. 448.
McDougall, B., and Gibson, F. (1958): Austral. J. exp. Biol., 36, p. 245.
Pansy, F. E., Khan, P., Pagano, J. R., and Donovick, R. (1950): Proc. Soc. exp. Biol. N.Y., 75, p. 618.
Wolfe, A. D., and Hahn, F. E. (1960): Bact. Proc., p. 177.

THE EFFECT OF A CHANGE IN SHAPE OF THE CARDIAC ACTION POTENTIAL ON THE IONIC CONDUCTANCES

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(*Accepted for publication 20th February, 1961.*)

SUMMARY.

Rabbit ventricular muscle was stimulated at a low constant rate and an extra stimulus was interposed shortly after every fourth action potential. Pairs of differently shaped action potentials were obtained in this way.

The magnitude and time course of polarisation resistance have been determined throughout these action potential pairs. The increase in polarisation resistance during the repolarisation phase was more prolonged and somewhat greater in the action potential of longer duration and area.

Changes in membrane conductance to sodium and potassium ions throughout the action potentials have been computed. Throughout the whole of the repolarisation phase the computed potassium conductance is lower and the computed sodium conductance is higher than in the resting state.

INTRODUCTION.

In dog Purkinje fibres (Weidmann, 1952) and rabbit ventricular fibres (Johnson and Tille, 1960) the membrane resistivity, after falling to a low value during the upstroke of the action potential, rapidly increases during the repolarisation phase to a value higher than the resting value. These findings support the hypothesis that the repolarisation phase of cardiac muscle is associated with a drop in membrane conductivity to potassium ions.

In this paper we have computed from the available experimental results, subject to certain assumptions, the time course of the membrane conductivity to sodium and potassium ions throughout the repolarisation phase.

The paper is divided into two parts. The first part describes a method of obtaining two differently shaped action potentials from the same fibre within 200 msec. of each other and also describes the time course of polarisation resistance throughout the two action potentials. The second part is devoted to the theoretical computation of the changes in ionic conductance of the membrane.

METHODS.

The apparatus, technique and procedure of determining the changes in polarisation resistance during the repolarisation phase have been described previously (Johnson and Tille, 1960, 1961).

RESULTS.

All the experiments reported in this paper were carried out on the one type of ventricular fibres which we have previously designated P fibres (Johnson and Tille, 1961). The electrical properties of the other kind of ventricular fibres (V fibres) are not suitable for the determination of polarisation resistance during the repolarisation phase.

Changes in membrane resistance associated with changes in action potential shape.

When cardiac tissue is stimulated at a constant rate all action potentials are identical in shape. If the constant rate is replaced by some rhythmic figure which is repeated at regular intervals the action potentials comprising each rhythmic figure will, in general, differ in shape. However, the shapes of action potentials in corresponding positions in all figures will be identical.

When rabbit ventricular muscle is driven at a low constant rate (e.g. 0·2 sec.$^{-1}$) and an extra stimulus is applied shortly after one of the stimuli, two action potentials of different shape are produced (Gibbs and Johnson, 1961).

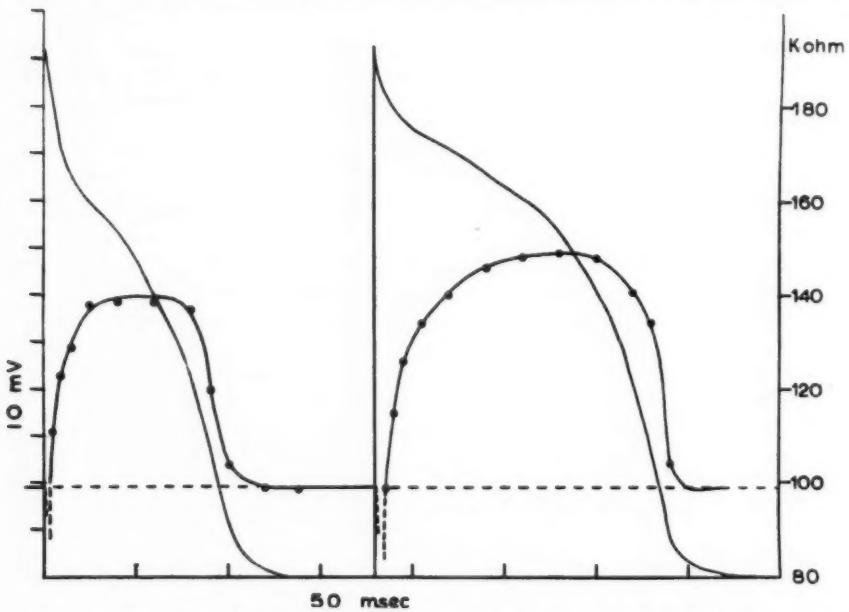

Fig. 1. The magnitude of polarisation resistance ● shown in time relationship to two dissimilarly shaped action potentials recorded from the same fibre. The action potential to the left was in response to one of stimuli applied at a constant rate (0·2 sec.$^{-1}$) and the other to an extra stimulus applied at the time shown in the abscissa. The horizontal interrupted line indicates the resting value of polarisation resistance.

We thus have a preparation in which two action potentials which differ in shape can be produced in the same fibre within 200 msec. of each other. By introducing this extra stimulus at a constant time after every fourth action potential a series of identical action potential pairs can be produced.

The changes in polarisation resistance throughout such pairs of action potentials were determined. Results typical of those which were obtained are shown in Fig. 1. In each of twenty fibres examined in this way the increase in polarisation resistance was more prolonged and somewhat greater in the case of the action potential of larger area and duration. No significant difference was detected in the value of resting polarisation resistance determined immediately before the upstrokes of the two action potentials.

Changes in membrane ionic conductance during the repolarisation phase.

In squid axon the action potential is generated by transient changes in conductance of the fibre membrane to sodium and potassium ions. If one were to suppose that the cardiac action potential is generated in a similar way it would be possible, making certain assumptions, to compute the changes in membrane conductance to these two ions throughout a given cardiac action potential.

During the repolarisation phase the membrane potential is changing very slowly with time and the interior of the fibre is almost equipotential at any time. We therefore assume that the changes in the longitudinal current are small enough to be neglected and the net membrane current is therefore zero. In this case, assuming that the action potential is generated by sodium and potassium ions and neglecting the leakage current:

$$-C_m \frac{dV}{dt} = I_K + I_{Na} \quad \ldots \quad (1)$$

$$I_K = (V - V_K) g_K \quad \ldots \quad (2)$$

$$I_{Na} = (V - V_{Na}) g_{Na} \quad \ldots \quad (3)$$

and

$$\frac{1}{R_m} = p_K + p_{Na} \quad \ldots \quad (4)$$

where V is the transmembrane potential, V_K and V_{Na} are the potassium and sodium equilibrium potentials (volts), I_K and I_{Na} are the potassium and sodium currents (Amps cm.$^{-2}$), g_K and g_{Na} are the chord conductances of potassium and sodium ions (mhos cm.$^{-2}$) and p_K and p_{Na} are the slope conductances of potassium and sodium ions (mhos cm.$^{-2}$). C_m is the membrane capacitance per unit area (Farads cm.$^{-2}$) and R_m is the specific membrane resistance (Ω cm.$^{-2}$).

The relationship between R_m and the polarisation resistance R_p in a simple leaky cable is given by $R_m \propto R_p^2$. For a hexagonal array of leaky cables which can be said to approximate the case of a syncytium (George, 1961):

$$R_m = kR_p^a \quad \ldots \quad (5)$$

where k depends on the structure of the fibre and is approximately constant for moderate changes in R_m and a has a value between 2 and 4.

Since R_p, as measured, is a slope resistance then $1/R_m$ determined from equation (5) must formally be equated with the slope conductances of potassium and sodium ions in equation (4).

In order to solve these equations (1 to 5) we made the assumption that the chord and slope conductances of each ion are equal and thus we can rewrite equation (4) as:

$$1/R_m = g_{Na} + g_K \quad \ldots \quad (4a)$$

This assumption is not unreasonable since in most cases the measured membrane polarisation resistance (and therefore R_m) remained constant over a large voltage range (including V_K) at any time after the upstroke.

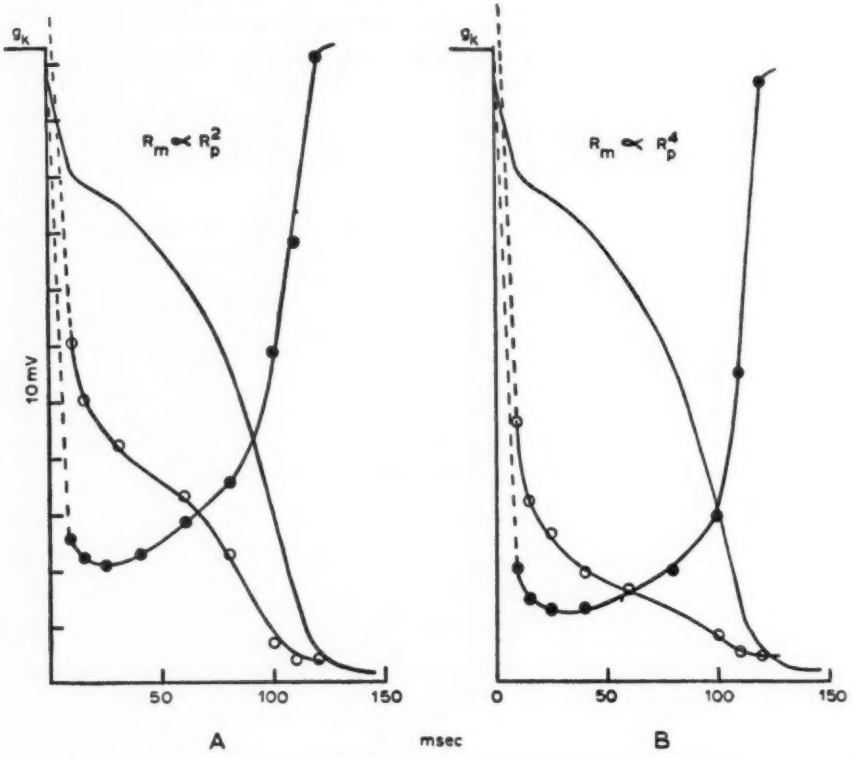

Fig. 2. The computed time course of the membrane conductivity to sodium ○ and potassium • ions throughout an action potential for the two limiting cases of $R_m \propto R_p^2$ (A) and $R_m \propto R_p^4$ (B). The conductances are plotted on a linear scale in arbitrary units; the relative magnitude of the units for the two cases was adjusted so that the resting potassium conductance (marked g_K) was at the same point on the ordinance scale for both cases.

The five simultaneous equations (1), (2), (3), (4a) and (5) were solved for g_K, g_{Na}, I_K, I_{Na} and R_m for the two limiting values of a ($a = 2$ and $a = 4$) using experimentally determined values for V, dV/dt, (R_mC_m) and R_p and taking V_{Na} as + 125 mV and V_K as − 2mV with respect to the resting potential. The computed conductances are expressed in arbitrary units since their absolute magnitude depends on the proportionality constant k of equation (5) which we could not determine.

Fig. 2b shows the results of such a computation for a typical action potential taking $R_p^4 \propto R_m$. The sodium conductance after rising to a high value during the upstroke falls rapidly to a value about ten times greater than the resting value. Thereafter it declines more slowly and finally returns to its resting value in the terminal phase of repolarisation. The potassium conductance is seen to fall abruptly immediately after the upstroke. It remains at a low value throughout the major part of the repolarisation phase and returns quickly to its original resting value at the end of the repolarisation phase.

These characteristic features of the time course of the two conductances were found in all our computations. Only their magnitude was found to change whilst the characteristic shapes of the two conductance time courses were retained. Fig. 2 shows the computed conductance time courses for the same action potential for the two limiting cases where R_m was taken to be proportional to Rp^2 and Rp^4. The general characteristic features of the two conductance time courses are the same in both cases.

There is a limit to the ability of the method to accurately follow the increase in polarisation resistance following the upstroke. This limit depends on the time required for recharging the membrane capacitance and the redevelopment of the IR drop across the membrane following its collapse during the rising phase (Johnson and Tille, 1961). The time constants of the fibres which we have examined were about 4 msecs. and the values of polarisation resistance obtained at short intervals (< 10 msec.) after the upstroke will therefore be under-estimated. Thus the fast drop of the two conductances may occur at an even earlier time than indicated in the figure.

Similar computations were carried out with the results obtained from the action potential pairs described in the first part of this paper. Fig. 3 shows a typical example of the conductance time courses of two differently shaped action potentials obtained from the same fibre by introducing an extra stimulus at a rate of $0{\cdot}2$ sec.$^{-1}$ Three characteristic differences in the conductance time courses of the two action potentials are revealed: (1) the return of both conductances to their resting value is delayed in the longer of the two action potentials; (2) at any membrane potential *both* conductances are lower in the longer of the two action potentials; (3) the initial drop in potassium conductance is always more rapid in the second action potential.

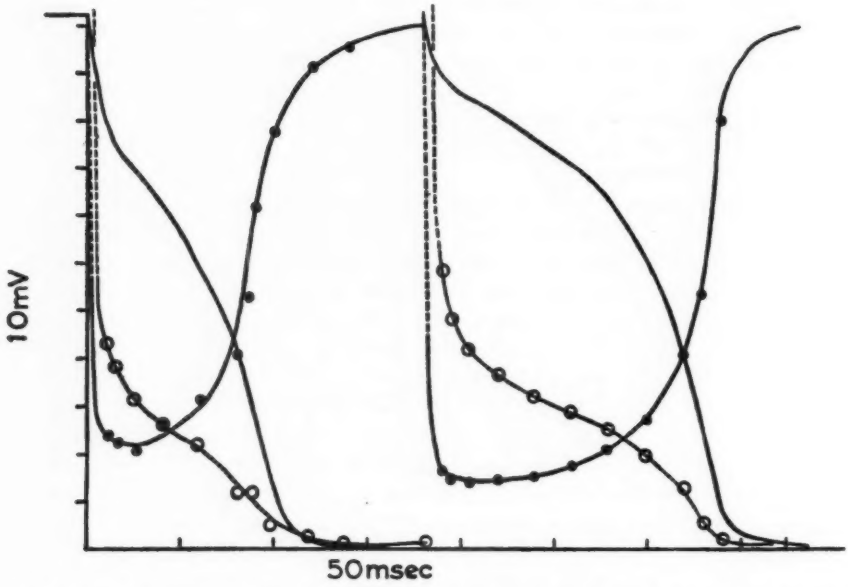

Fig. 3. The computed time course of the membrane conductivity to sodium ○ and potassium ● ions throughout the two differently shaped action potentials shown in Fig. 1 ($R_m \propto R_p^2$). The conductances are plotted on a linear scale in arbitrary units; the resting value of potassium conductance is indicated by the horizontal bar at the top of the ordinate scale.

DISCUSSION.

Solving a modified set of the Hodgkin-Huxley equations for squid axon Noble (1960) was able to produce cardiac-like action potentials. The major modifications were a reduction of the Hodgkin and Huxley maximum potassium conductance $\bar{g}_K$ to one-fiftieth of that in nerve, together with an increase in its time constants, and the inclusion of an instantaneous voltage dependent fall in g_K. Some modifications were also applied to the equations describing g_{Na}. The changes in membrane ionic conductances which are associated with his action potentials bear a very close resemblance to those which we have computed from our experimental results.

However, there is one important distinction. In Noble's model both g_K and g_{Na} are voltage dependent and regenerative repolarisation is easily achieved by hyperpolarisations of more than 30 mV. We find (Johnson and Tille, 1960) that in fibres of the rabbit right ventricle the net membrane conductance during the repolarisation phase appears to be independent of membrane potential. No non-linearity was observed in the relationship between hyperpolarising current

and voltage displacement during this phase. No regenerative repolarisation was observed with hyperpolarisations of up to 80 mV.

We have described a situation in which two action potentials initiated in the same cell within a few hundred milliseconds of each other and with identical membrane resting potentials and membrane resting resistances just before their upstrokes differ markedly in the time course of repolarisation. In general, the shape of the repolarisation phase of a rabbit ventricular action potential is determined in some way by the action potentials preceding it (Gibbs and Johnson, 1960). It seems unlikely, therefore, that an adequate hypothesis about the behaviour of the two conductances during the repolarisation phase can be based entirely on variables which are instantaneously or nearly instantaneously voltage dependent (e.g. the Hodgkin and Huxley equations, the time constants of which are of the order of a few milliseconds).

The possibility that the drop in g_K is a voltage independent, all-or-none response the magnitude and duration of which is in some way determined by the preceding stimulation pattern should be investigated.

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REFERENCES.

George, P. (1961): Aust. J. exp. Biol. (in press).
Gibbs, C. L., and Johnson, E. A. (1961): Circ. Res., 9, p. 165.
Johnson, E. A., and Tille, J. (1960): Aust. J. exp. Biol., 38, p. 509.
Johnson, E. A., and Tille, J. (1961): J. gen. physiol., 44, p. 443.
Noble, D. (1960): Nature, 188, p. 495.
Weidmann, S. (1952): J. Physiol., 118, p. 348.

THE DYNAMIC RED CELL STORAGE FUNCTION OF THE SPLEEN IN SHEEP

III. RELATIONSHIP TO DETERMINATION OF BLOOD VOLUME, TOTAL RED CELL VOLUME, AND PLASMA VOLUME

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SUMMARY.

In the sheep the spleen has been shown to act as a dynamic reservoir of highly concentrated blood. As the number of red cells retained in the spleen is dependant upon the emotional state of the animal at the time, the values of the jugular haematocrit and of the F_{cells} ratio are subject to wide variations. The extent to which such variations can affect the calculated values of blood volume (BV) and red cell volume (RCV) from plasma volume (PV) and jugular haematocrit has been demonstrated. Separate determination of RCV by labelled-cell techniques, such as ^{51}Cr-labelling, may also be fallacious, because a considerable proportion of the total red cells (of the order of 10 p.c.) was found to be withheld from free "mixing" in some sheep; but this was readily overcome by the intravenous administration of adrenaline. Little error could result, and considerable saving of time could be made, by basing estimates of RCV and PV on a single sample taken 10 minutes after administration of labelled cells and T-1824 together with adrenaline to contract the spleen and thereby promote rapid and complete "mixing". More simply, after injection of only T-1824 plus adrenaline, BV_{pl} could be calculated from the formula $PV/(100 - H \times F_{cells})$ where H is the haematocrit determined after 2-3 minutes, PV is based on a single plasma sample at 10 minutes and F_{cells} after adrenaline is taken to be 0·81.

The mean PV of 21 determinations on 13 Merino sheep, as determined by the T-1824 method, was 44·6 ml./kg. (S.D., 3·1 ml./kg.). The mean BV, RCV and PV of 5 sheep, as determined by ^{51}Cr-labelled cells and T-1824, were 66·4, 19·7 and 46·7 ml./kg. respectively (S.D., 5·4, 2·8 and 3·6 ml./kg. respectively).

INTRODUCTION.

It was reported in Paper I of this series (Turner and Hodgetts, 1959) that the jugular haematocrit of a sheep might vary over a wide range depending upon whether the animal was excited or tranquil. This raises the question of the reliability of estimations of red cell volume (RCV) and of blood volume (BV_{pl}) in this species by plasma-haematocrit methods. Furthermore, it was suggested that in some sheep prolonged sequestration of a significant proportion

of the total red cells might occur in the spleen so that estimations of these volumes by isotope-labelled red cell dilution methods might also be subject to error.

In this communication these problems are discussed further, and data on plasma volume (PV), blood volume (BV) and RCV are presented.

Methods and Material.

Where not otherwise stated these were as described in Paper I (Turner and Hodgetts, 1959) and Paper II (Turner and Hodgetts, 1960). The determination of RCV was based on the ^{51}Cr-labelled red cell dilution method of Mollison and Veall (1955) and PV was determined by the T-1824 dilution method as described by Chinard (1951). Packed blood cells were labelled with ^{51}Cr as sodium chromate (specific activity of the order of 10 μc./1 μg. chromium). With acid-citrate-dextrose (ACD) as the anticoagulant, uptake ranged from 80-96 p.c. in 30 minutes at room temperature (12-14° C.). In some sheep, lysis of red cells in the presence of ACD necessitated the use of heparin as anticoagulant; in these cases the uptake was slower, e.g. 36 p.c. after 30 minutes at room temperature, 75 p.c. after 60 minutes and 88 p.c. after 90 minutes. Washed labelled cells were suspended in a 2 p.c. ACD plasma-saline solution immediately prior to injection and a sample was removed for determination of its activity. A 20 ml. sample of the cell suspension was taken for injection, the remainder was spun rapidly and the activity in the supernatant was determined. It was normally of the order of 0·4 p.c.

Intrajugular polythene cannulae, containing heparinized saline, were established in both veins. Blood samples were removed through one cannula and labelled cells, dye or adrenaline were introduced through a second by means of a three-way metal tap. Either one 15 ml. pre-injection blood sample was collected or, in animals with residual activity in the circulating blood, three samples were taken. The suspension of labelled cells (4·6-7·3 ml. of cells containing 7·6-20·4 μc./ml. of cells) was injected from a calibrated syringe (washout technique). Two minutes later 3 ml. of a freshly prepared filtered T-1824 solution (50 mg./10 ml.) in saline was introduced. 8 ml. blood samples were collected at approximately 10-minute intervals for a 60-100-minute period. Usually adrenaline (0·5 ml. 1 : 1000) was injected following the 40- or 70-minute sample and an additional sample was collected 2-3 minutes later. ^{51}Cr activity, T-1824 concentration, haematocrit* and plasma protein concentration were determined on all samples; 5 ml. of blood was spun, the plasma was used for the determination of T-1824 concentration by the acetone extraction method, and the residual cells were diluted to 10 ml. with water in a standard flask, saponin was added, and ^{51}Cr activity was measured in a gamma-ray scintillation counter. Activity of a solidified ^{51}Cr standard and background activity were measured before and after the determination of the activity of each sample, and samples were corrected for decay, background activity and instability of the counting equipment. Rate of loss of ^{51}Cr from red cells was approximately 7 p.c. in the 60 minutes following injection when the cells were labelled in the presence of ACD or heparin for 30 minutes and did not significantly differ between intact and splenectomized sheep. Loss of activity was approximately 20 p.c. in 4 hours and 45 p.c. in 24 hours; 20-25 p.c. activity still remained in the circulation after 1 week, at which time approximately a 5 p.c. daily loss was occurring. These results indicate a slightly higher loss than that reported by Drury and Tucker (1958). The rate of loss was related to the time of labelling; labelling for 60 minutes was followed by 11·5 p.c. loss and for 90 minutes by 13 p.c. loss in the 60 minutes following injection.

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* Total packed cells, including leucocytes, corrected for trapped plasma.

RESULTS.

Results of plasma volume determinations.

The results of 21 determinations on 13 Merino sheep, ranging in weight from 30·0 kg. to 48·0 kg., were as follows: PV 40·6-52·9 ml./kg. The mean was 44·6 ml./kg., with a S.D. of 3·1 ml./kg. Prof. W. V. Macfarlane has used these data earlier to compare them with values obtained in sheep in hot environments (Macfarlane, Morris, Howard and Budtz-Olsen, 1959).

Influence of fluctuations of jugular haematocrit upon estimates of blood volume by the T-1824-haematocrit method.

In view of the fluctuations of jugular haematocrit reported in Paper I, it follows that estimates of BV_{pl} and of RCV must also be subject to fluctuations in response to various degrees of excitement. In Table 1 these estimates are calculated for a hypothetical "average" sheep, by using for PV the mean value of 44·6 ml./kg., and the means of the jugular haematocrits given in Paper I. The four sets of conditions for which calculations have been made are (a) placidity induced in receptive sheep by gently restraining and soothing them ("holding"); (b) moderate but not excessive excitement, as observed in untrained sheep subjected to experiment; (c) complete isolation from external stimuli during anaesthesia by Pentobarbitonum Sodium (PBS); and (d) simulated excessive excitement and splenic contraction induced by i.v. injection of adrenaline.

TABLE 1.

Effect of fluctuations of jugular haematocrit upon BV_{pl} and RCV of a hypothetical "average" sheep under various conditions.

State of sheep	Jugular haematocrit	BV_{pl}* (ml./kg.)	RCV (ml./kg.)
Untrained	36	69·7	25·1
Tranquillized by "holding"	31	64·6	20·0
Anaesthetized by PBS	25	59·5	14·9
2-3 min. after adrenaline i.v.	40	74·3	29·7

* Assuming the mean value for PV = 44·6 ml./kg.

For this hypothetical example, the calculated BV_{pl} differed, depending whether the animal was tranquil or moderately excited, by an amount representing 3.8 p.c. of the mean of the two values, and the RCV differed by 11·3 p.c. of the mean; with the extreme values (haematocrit during anaesthesia compared with that after adrenaline) differences were greater still, the estimates being 11·1 p.c. and 32·2 p.c. on either side of the mean of the two values. In individual sheep some quite large differences would be possible: e.g., for sheep 751 (Table 1 in Paper I) the jugular haematocrit ranged from 26·6 in the

tranquil state to 35·2 in the moderately excited state; if for its PV the mean value 44·6 ml./kg. is used, it can be calculated that BV_{pl} would vary by 6·2 p.c. on either side of the mean of the two values for this animal, while RCV would vary by 19·8 p.c.

It is clear, therefore, that this method is inadequate for sheep, because of the instability of jugular haematocrit and the consequent difficulty of establishing a "normal" value for it.

The F_{cells} factor in relationship to blood volume estimation in sheep.

Plasma-haematocrit methods have another inadequacy which is not peculiar to sheep and other species with unstable haematocrits. This is a consequence of the rheological fact that the haematocrit in large blood vessels is always greater than that in small vessels. For species in which the red cell storage function of the spleen is unimportant, the average haematocrit of all the blood in the body is therefore less than that of jugular blood, the ratio of total-blood haematocrit to jugular-blood haematocrit being virtually constant, characteristic for the species, and independent of the actual numbers and mean concentrations of red cells in the blood. For example, in normal healthy man this ratio, referred to by Reeve *et al.* (1953) as the F_{cells} ratio, is 0·91, and multiplication of the jugular haematocrit (corrected for trapped plasma) by this figure allows the haematocrit to be used with some confidence for estimating BV and RCV when PV is known. It has been shown by Reeve *et al.* that this holds also for splenectomized dogs, a species in which the cell storage function of the spleen is important: in 16 such dogs the mean value of F_{cells} was $0·899 \pm 0·023$ (S.D.). On the other hand, with 9 intact dogs the mean value was higher (0·981) and the range of values greater (S.D., 0·057), the least value (0·861) being found in an emotionally tense dog and the greatest (1·078) in the most placid dog. With 5 dogs anaesthetized with PBS the F_{cells} value was still higher with a mean of 1·100. Obviously, since F_{cells} is the ratio of total-blood haematocrit to jugular-blood haematocrit, F_{cells} will vary inversely with the jugular haematocrit when the total-blood haematocrit remains constant, as it does when total red cell numbers and PV remain unchanged. During estimations of BV by separate determinations of PV (T-1824) and of RCV (^{51}Cr-labelled red cell dilution method), data on the F_{cells} factor in sheep were obtained under various conditions.

The sheep had been chosen for their placidity (jugular haematocrits 21·9-26·4, mean 24·3) so that they would not become restive under the prolonged restraint during the taking of repeated blood samples. A wide range of emotional states and of jugular haematocrits is therefore not represented. Analysis of the data shows that in splenectomized sheep the F_{cells} ratio was low, the mean value for 6 animals being $0·832 \pm 0·027$: in intact sheep it was greater and more variable, the mean value for 5 animals being $1·222 \pm 0·092$. Following adrenaline injection to maximally contract the spleen the F_{cells} fell to low values,

TABLE 2.

F_{cells} ratio in intact, splenectomized and adrenaline-treated sheep.

Sheep no.	Total blood haematocrit (a)	Jugular haematocrit (b)	Jugular haematocrit after adrenaline (i.v.) (c)	F_{cells} ratio: Intact a/b	F_{cells} ratio: Adrenaline treated a/c	F_{cells} ratio: Splenectomized a/b
822						
Intact	34·3	26·4	42·9	1·30	0·80	
904						
Intact	28·4	24·7	35·7 (36·2*)	1·15	0·80	
885						
Intact	28·9	21·9	35·8	1·32	0·81	
Splenectomized 2½ hr.	21·7	27·2				0·80
Splenectomized 1 week	18·2	21·1				0·86
895						
Intact	29·2	26·3	37·0 (37·7*)	1·11	0·79	
Splenectomized 2½ hr.	26·8	33·6				0·80
910						
Intact	27·3	22·2	32·3	1·23	0·85	
Splenectomized 2 hr.	22·1	26·0				0·85
590						
Splenectomized 3 years 7 months	19·4	22·7				0·85
854						
Splenectomized 9 months	20·1	24·4				0·82
Mean				1·222±0·092	0·810±0·023	0·832±0·027

* Haematocrit before adjustment for water shift.

the mean value for 5 animals being $0{\cdot}810 \pm 0{\cdot}023$, which was not significantly different ($0{\cdot}20 < p < 0{\cdot}30$) from that of splenectomized sheep. It is likely that in very excitable sheep (e.g. sheep 499, Table 2, Paper I) low values approaching these might well be reached.

These data illustrate the difficulty of using plasma-haematocrit methods for determining BV_{pl} and RCV in sheep, even when an attempt is made to allow for the F_{cells} factor, since it is impossible to predict with any accuracy what the value of this might be except in splenectomized or adrenaline-injected sheep. For example, using the mean values recorded in Paper I, consider a sheep taken at random with jugular haematocrit of 36. Assuming the mean value for PV (44·6 ml./kg.), the estimate for BV_{pl} if F_{cells} were 0·83 would be $44{\cdot}6/(100\text{-}36 \times 0{\cdot}83)$, or 63·7 ml./kg.; if F_{cells} were 1·22, it would be 79·5 ml./kg., an 11·0 p.c. variation around the mean of the two values. Or consider the "average" placid sheep with a jugular haematocrit of 31; the corresponding estimates would be 60·0 ml./kg. and 71·7 ml./kg., an 8·9 p.c. variation around the mean of the two values.

Influence of incomplete "mixing" in the spleen.

It is clear from the foregoing that reliable estimates of RCV and BV in sheep can be made only by separately determining PV and RCV by means of suitable techniques. However, the validity of this rests on the assumption that all the red cells in the spleen are in dynamic equilibrium with the circulating blood and that a state of complete displacement and replacement of all red cells in the spleen has occurred when the estimate of RCV is made (i.e. when the activity curve becomes linear). Reservations concerning this assumption, at least in sheep during PBS anaesthesia, were made when interpreting the data in Fig. 1, Paper I, since an increased uptake of ^{51}Cr-labelled cells in the spleen occurred after injection of adrenaline. During a determination of BV by the ^{32}P-labelled red cell dilution method, Reeve *et al.* had also observed in one out of five PBS-anaesthetized dogs that the ^{32}P-level fell by 8 p.c. of its previous value after adrenaline was injected; this they attributed to "incomplete mixing of labelled cells in this animal". Again, Sliwinski and Lilienfield (1958) noted that in anaesthetized dogs mixing might not be complete for up to 90 minutes.

During estimations of RCV and BV, we found that incomplete mixing may occur also in conscious sheep (Fig. 1). In sheep 883 complete mixing appeared to have been reached 30-40 minutes after the administration of labelled cells as the activity curve had by then become linear and continued so for about 30 minutes; yet the injection of adrenaline caused a sudden fall of activity, indicating the entry of additional unlabelled cells into the circulating blood, after which the activity curve again became linear with the same slope as previously. Extrapolation of the second part of the activity curve led to an estimate of RCV of 744 ml., whereas the first part corresponded to 664 ml., i.e. 80 ml. of red cells (10·8 p.c.) had been withheld in the spleen, in virtual isolation from the circu-

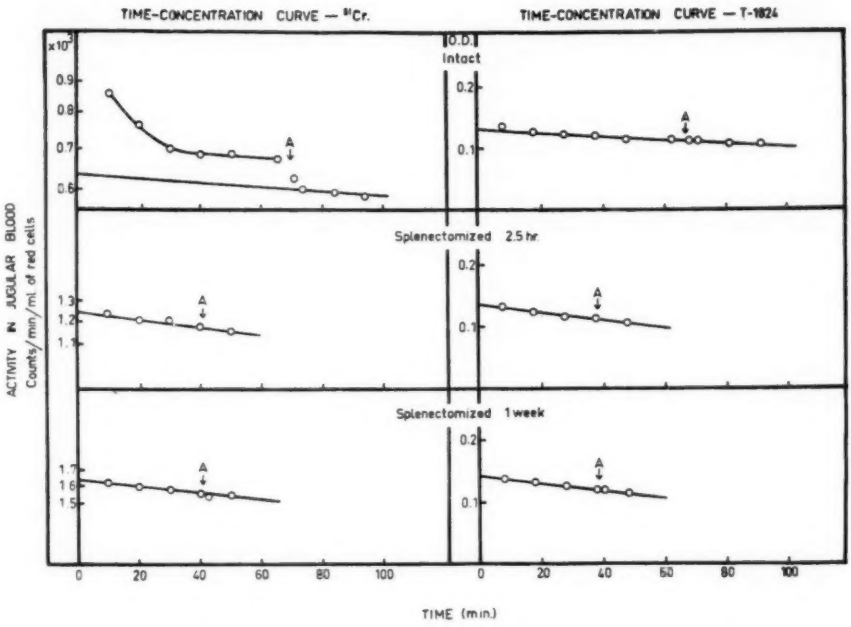

Fig. 1. Curves for the determination of RCV and PV in sheep 885 before splenectomy, 2·5 hr. afterwards and 1 week afterwards. At A, adrenaline (12·5 μg./kg.) was injected.

lating blood, for at least 30 minutes. In sheep 822, also a particularly placid sheep, the two estimates were 1021 ml. prior to adrenaline, and 1120 ml. after it, indicating that 99 ml. (8.8 p.c.) of the total red cells had been thus sequestered from the circulation. In neither sheep was the release of these sequestered red cells accompanied by any significant change in the T-1824 concentration curve, indicating that this was not accompanied by release of much plasma; this is in agreement with the findings reported in Paper I, that the haematocrit of splenic "blood" in sheep may be very high (up to 90 p.c.). Both these sheep probably had large spleens. When the second animal was later splenectomized under PBS anaesthesia, the jugular haematocrit at the moment of ablation was still low (25 p.c.); the spleen weighed 645 g. (1·37 p.c. of the body weight) and contained 391 ml. of thick blood of which 344 ml. were cells.

Anomalous results were obtained with one sheep, both before and after splenectomy. On each occasion lysis of red cells occurred when they were placed in ACD solution and hence heparin had to be used. Because labelling was slower under these conditions, it was continued for 90 minutes to encourage a high uptake of ^{51}Cr. On each occasion the rate of loss of ^{51}Cr after injection

was similar (12-13 p.c. during the first 60 minutes). However, when adrenaline was injected at the completion of the BV estimation there was an increase of activity per ml. of cells, indicating that labelled cells had entered the circulation from some depot, the nature of which was not determined. There was no increase in thickness of the "buffy coat" to suggest an increased number of leucocytes which might carry ^{51}Cr; hence the possibility must be considered that unstable, possibly abnormal, labelled red cells had accumulated in some part of the body, and had been displaced as a result of the action (e.g. increased blood flow) of adrenaline. The data on PV and RCV for this animal were computed from the curves before adrenaline injection.

Results of red cell volume and blood volume determinations.

For the 5 sheep investigated, the data for RCV, PV and BV are given in Table 3. The RCV ranged from 16·3 to 23·8 with a mean of $19·7 \pm 2·8$; and the BV from 73·8 to 59·7 with a mean of $66·4 \pm 5·4$.

TABLE 3.

Values for red cell volume (RCV), plasma volume (PV), and blood volume (BV).

Sheep No.	RCV ml./kg.	PV ml./kg.	BV ml./kg.	$BV_{p.i.}$* ml./kg.
822	23·8	45·6	69·4	68·9
904	20·9	52·9	73·8	73·3
885	18·6	45·7	64·3	63·0
895	18·9	45·8	64·7	63·7
910	16·3	43·4	59·7	58·8
Mean	$19·7 \pm 2·8$†	$46·7 \pm 3·6$	$66·4 \pm 5·4$	$65·5 \pm 5·6$

* Calculated from PV, based on 8 minute sample, and jugular haematocrit following adrenaline i.v.

† Standard deviation.

DISCUSSION.

These findings emphasize the important rôle of the sheep's spleen as a dynamic reservoir of red cells; indeed, the large values for F_{cells} under tranquil conditions in some sheep and the finding that in the conscious state "mixing" may be incomplete after 70 minutes suggest that this function is developed in some sheep to an even greater degree than in many dogs. It is clear that a considerable error is likely to occur if BV is estimated from PV and the jugular haematocrit and that reliable results will be obtained only if RCV is separately estimated; even then, because of the possibility of prolonged sequestration of considerable numbers of red cells in the spleen of some sheep, especially sheep with large spleens and a placid temperament, it would seem desirable as a routine to contract the spleen by means of adrenaline.

In the standard procedure for determining blood volume in sheep it is necessary, because of the slow "mixing", the variation in the period required for

completion of this process and the high rate of loss of ^{51}Cr from the red cells, to withdraw samples over a long period of time (60-90 minutes); in man, a species in which the red cell storage function of the spleen is unimportant, a single sample taken after an interval of 10 minutes is often considered adequate. It would seem that this latter method might well be applied to sheep and other animals in which the spleen is important (ruminants, horses, dogs) by taking advantage of the spleen contracting effect of adrenaline; indeed, this suggestion has already been made by Anderson and Rogers (1957) with reference to goats. For example, in the sheep, injection of T-1824 and ^{51}Cr-labelled cells could be combined with adrenaline injection (say, 30 μg./kg); a blood sample could be collected after 10 minutes with every confidence that mixing was complete and that no important loss of T-1824 or ^{51}Cr had occurred. Another simplification of technique becomes possible by taking advantage of the relatively constant F_{cells} ratio ($0{\cdot}81 \pm 0{\cdot}023$) observed in sheep injected with adrenaline to maximally contract the spleen. By combining adrenaline with T-1824, the maximum haematocrit (corrected for trapped plasma and if necessary adjusted for water shift) could be determined from a sample taken 2-3 minutes later, and the dye-concentration from a sample taken after 10 minutes; BV_{pl} could then be calculated to a reasonable approximation from PV, maximum jugular haematocrit (H) and a value for F_{cells} of $0{\cdot}81$ by the formula $BV_{pl} = PV/_{(100-H\times 0.81)}$. The BV_{pl} values, calculated in this way, are compared in Table 3 with BV determined directly by separate estimation of PV and RCV. Further work on the evaluation of and error in F_{cells} in adrenaline-treated sheep may be desirable but moderate change in its value would cause only slight change in BV_{pl}; for example, for a jugular haematocrit after adrenaline of 40 (the mean value in Paper I) an increase of F_{cells} from $0{\cdot}81$ to $0{\cdot}85$ (approximately 5 p.c.) would cause BV_{pl} to increase by only $2{\cdot}4$ p.c.

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REFERENCES.

Anderson, R. S., and Rogers, E. B. (1957): Amer: J. Physiol., 188, p. 178.
Chinard, F. P. (1951): "Methods in Medical Research", 4, p. 38.
Drury, A. N., and Tucker, E. M. (1958): Immunology, 1, p. 204.
Mollison, P. L., and Veall, N. (1955): Brit. J. Haemat., 1, p. 62.
Macfarlane, W. V., Morris, R. J. H., Howard, B., and Budtz-Olsen, O. E. (1959): Aust. J. agric. Res., 10, p. 269.
Reeve, E. B., Gregersen, M. I., Allen, T. H., and Sear, H. (1953): Amer. J. Physiol., 175, p. 195.
Sliwinski, A. J., and Lilienfield, L. S. (1958): Proc. Soc. exp. Biol., N.Y., 90, p. 648.
Turner, A. W., and Hodgetts, V. E. (1959): Aust. J. exp. Biol., 37, p. 399.
Turner, A. W., and Hodgetts, V. E. (1960): *Ibid.*, 38, p. 79.

THE ANALYSIS OF ELECTROPHORESIS PATTERNS: A COMPARISON OF TWO METHODS WITH THE AID OF RAYLEIGH INTERFERENCE OPTICS

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SUMMARY.

Two methods of serum electrophoresis pattern resolution have been investigated:

(a) the method of Kabat and Tiselius (1939) in which the components are delimited by dropping perpendiculars from minima in the gradient curve;

(b) the method of Svedberg and Pedersen (1940) in which the pattern is resolved into Gaussian curves, the sum of whose ordinates at any given point is equal to the ordinate of the gradient curve at that point.

It was found that the latter method was the less influenced by the degree of separation of the components but it is inconvenient to use with interferometric optical systems because it is necessary to plot the entire gradient curve.

It was found possible to use nearly all the cell height of the Perkin-Elmer model 38A electrophoresis instrument if the plateholder were modified to ensure reproducible location of the plate at each exposure. This made it possible to carry out electrophoresis for a time which gave sufficient separation of the components to enable the method of Kabat and Tiselius to be used.

INTRODUCTION.

Two methods are commonly used for resolving complex moving boundary electrophoresis patterns where the separation of the individual boundaries is incomplete, as in serum. In one, due to Kabat and Tiselius (1939), ordinates are drawn to the baseline from the minima between adjoining peaks. In the other, due to Svedberg and Pedersen (1940), Gaussian curves are constructed under each peak so that the sum of their ordinates at any point is equal to the observed value of the electrophoresis curve.

The second is generally considered to give values less dependent upon the degree of separation of the components. The first is preferred by users of interference optics because it avoids the necessity of plotting a complete gradient curve from fringe separation measurements, as all that need be done is to

[1] Part of the expenses of this investigation were defrayed by a grant from the National Health and Medical Research Council, Australia.

determine the fringe number of the minimum between each peak, either from a superimposed schlieren gradient curve, or, more accurately, by numerical interpolation from the fringe separations about the minimum. The concentration of each component is proportional to the number of fringes between each minimum. Because of the increasing use of interference optical systems a revaluation of the two methods of resolution should be attempted, with particular emphasis on the effect of the degree of boundary separation. This paper presents the results of some studies on electrophoresis pattern-resolution, made with the aid of a photoelectric Rayleigh fringe comparator.

Experimental.

Apparatus.

The electrophoresis instrument was a Perkin-Elmer model 38A Tiselius apparatus, fitted with the phase-plate gradient Rayleigh interferometric optical system of Moore and Opperman (1956). The cell bath was kept at 2° C. by water circulated from a thermostatically controlled refrigerated tank.

The electrophoresis patterns were recorded on Kodak contrast process-orthochromatic plates. The plates were scanned with the aid of a recording photoelectric comparator. The construction and operation of this instrument have been described elsewhere (Curtain, 1960).

Methods.

Electrophoresis of serum diluted 1/5 was carried out in sodium veronal buffer, pH 8·60, $I = 0{\cdot}1$, at a potential gradient of 17·5 v. cm^{-1}. Photographic exposures were made at 1,500, 2,000, 3,000 and 4,000 seconds after the start of the run. The standard Perkin-Elmer 2 ml. cell was used. Gradient curves were plotted from the fringe separations on the comparator recorder chart.

The ascending and descending patterns of each run were resolved into the γ, β, α_2 and α_1 globulins and albumin by the method of Kabat and Tiselius (1939) (method a) and by the method of Svedberg and Pedersen (1940) (method b). The area under each curve was determined with the aid of a planimeter.

RESULTS.

The mean apparent percentages at the four exposure times from ten runs and their standard deviations are set out in Table 1. It can be seen that the values obtained by method (b) are less dependent upon the degree of separation than those of method (a). In both the ascending and descending limbs method (a) gives low mean values for the α_1 globulin at the shorter times compared with method (b) These α_1 globulin values increase until they are close to the values by method (b) at 4,000 sec.

The fact that the two sets of values approach each other with increasing separation suggested that increased duration of electrophoresis would improve the agreement between the two methods. Unfortunately the full height of the relatively short centre section (46 mm.) of the Perkin-Elmer cell cannot be used as the normal procedure with the interferometric optical system is to produce a final pattern with at least 6 mm. of horizontal fringes in front of the albumin and

TABLE 1.

The effect of time of electrophoresis on the mean apparent percentage composition of ten samples of different normal sera, as determined by the method of Kabat and Tiselius (a) and by the method of Svedberg and Pedersen (b). The figures in brackets in this and the following tables are standard deviations.

Time (sec.)		Ascending pattern					Descending pattern				
		γ	β	α_2	α_1	Albumin	γ	β	α_2	α_1	Albumin
1,500	(a)	12·1 (1·36)	14·8 (1·42)	10·9 (0·99)	3·7 (0·35)	58·5 (1·91)	13·1 (1·40)	15·2 (1·61)	11·1 (1·10)	3·9 (0·66)	56·7 (1·91)
	(b)	11·7 (1·23)	14·1 (1·36)	9·7 (0·93)	9·2 (0·47)	58·3 (2·13)	13·9 (1·71)	14·3 (1·92)	10·1 (0·96)	6·4 (0·67)	55·3 (1·83)
2,000	(a)	11·9 (1·29)	14·6 (1·39)	10·6 (0·96)	4·3 (0·41)	58·6 (2·0)	13·2 (1·40)	14·9 (1·71)	11·0 (1·01)	4·3 (0·65)	56·6 (1·85)
	(b)	11·1 (1·20)	14·2 (1·37)	9·9 (0·96)	6·3 (0·48)	58·5 (2·06)	13·7 (1·71)	14·4 (1·90)	10·0 (0·95)	6·5 (0·67)	55·4 (1·72)
3,000	(a)	11·8 (1·27)	14·4 (1·36)	10·3 (0·94)	4·9 (0·44)	58·6 (2·07)	13·5 (1·50)	14·6 (1·81)	10·4 (0·99)	4·9 (0·67)	56·6 (1·82)
	(b)	11·1 (1·20)	14·2 (1·37)	10·0 (0·93)	6·2 (0·47)	58·5 (2·10)	13·5 (1·61)	14·4 (1·92)	10·1 (0·95)	6·5 (0·68)	55·5 (1·76)
4,000	(a)	10·6 (1·26)	14·3 (1·35)	10·2 (0·93)	5·7 (0·46)	59·2 (2·10)	13·1 (1·66)	14·3 (1·91)	10·2 (0·96)	5·9 (0·69)	56·5 (1·89)
	(b)	11·3 (1·21)	14·2 (1·37)	9·9 (0·91)	6·2 (0·47)	58·4 (1·99)	13·4 (1·55)	14·4 (1·91)	10·1 (0·95)	6·5 (0·95)	56·5 (1·79)

TABLE 2.

Comparison of the average apparent percentage composition of eight sera by method (a) and method (b) after 6,000 sec. of electrophoresis under standard conditions.

Method	Ascending limb					Descending limb				
	γ	β	α_2	α_1	Albumin	γ	β	α_2	α_1	Albumin
Kabat and Tiselius	10·9	14·2	10·0	6·0	58·9	13·2	14·4	10·0	6·2	56·2
	(1·31)	(1·37)	(0·97)	(0·52)	(2·16)	(1·71)	(1·89)	(0·99)	(0·69)	(1·89)
Svedberg and Pedersen	11·0	14·2	10·0	6·1	58·7	13·4	14·4	10·0	6·4	55·9
	(1·27)	(1·39)	(0·96)	(0·49)	(2·05)	(1·61)	(1·96)	(0·93)	(0·71)	(1·78)

behind the concentration boundaries to facilitate alignment of the pattern in the comparator.

It was decided to modify the plateholder to ensure that the fringes, in the absence of a refractive index gradient in the cell, would always be at a right angle to the sides of the plate so that accurate alignment of the plate itself would suffice to align the pattern in the comparator. The plate has approximately 0·9 mm. clearance in the "Graflex" holder supplied with the Perkin-Elmer instrument, and the holder itself has approximately 1·3 mm. clearance in the camera back. These clearances were eliminated by lining the plate compartment with a layer of rubber dental dam, surfaced with a brass strip (bonded with "Laminex" contact cement) and by building up the outside of the plateholder with brass strips fixed with counter-sunk screws. Ten exposures were taken of an empty cell with the plates in the treated holder. On examination in the comparator the fringes were found to be parallel to the track of the instrument to within 4μ in 45 mm.

Eight normal sera were then submitted to electrophoresis under the standard conditions and the final exposures made at 6,000 sec. The average ratios of the two methods of resolution and the standard deviation for each of the components is given in Table 2. It can be seen that the methods of resolution agree very closely after 6,000 sec. of electrophoresis under the specified conditions.

To test the reproducibility of the method 5 samples of the same serum were submitted to electrophoresis for 6,000 sec. and the patterns analysed by determining the minima by numerical interpolation from the comparator chart and by counting the fringes between these minima. The results are given in Table 3.

TABLE 3.

Average percentage composition of five replicate runs of 6,000 sec. duration on the same serum. Values obtained by counting fringes between gradient curve minima determined by numerical interpolation from fringe spacing.

Ascending limb					Descending limb				
γ	β	α_2	α_1	albumin	γ	β	α_2	α_1	albumin
11·1 (1·07)	15·9 (0·96)	9·6 (0·99)	6·9 (1·01)	56·5 (0·98)	13·3 (0·99)	15·9 (1·09)	9·9 (1·05)	6·5 (1·17)	54·4 (0·96)

It can be seen that there is excellent agreement between the 5 analyses. The standard deviations are somewhat smaller than those reported by Moore (1956) for replicate analyses with the same optical system. This is probably due to the greater boundary resolution and to the use of numerical interpolation from fringe spacing to determine the limit of each component, for which Moore and Opperman used the phase-plate gradient curve.

REFERENCES.

Curtain, C. C. (1960): J. sci. Instrum., 37, p. 190.
Moore, D. H. (1956): Biochim. et biophys. Acta, 22, p. 141.
Moore, D. H., and Opperman, K. O. (1956): *Ibid.*, 22, 136.
Svedberg, T., and Pedersen, K. O. (1940): "The Ultracentrifuge", Oxford Univ. Press (London).
Tiselius, A., and Kabat, E. A. (1939): J. exp. Med., 69, p. 119.

THE QUANTITATIVE ELECTROPHORESIS OF SERUM PROTEINS IN THE PRESENCE OF HAEMOGLOBIN

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SUMMARY.

A method for correcting serum electrophoresis patterns for the presence of haemoglobin is described. The electrophoresis pattern of the serum is photographed first in red light at 690 mμ, then the cylindrical lens of the instrument is removed and the direct image of the cell is photographed in green light at 546 mμ. The distribution of haemoglobin along the cell is determined by densitometry of the direct image negative which gives the integral of that part of the serum gradient curve due to haemoglobin. Two points of inflection occur in the densitometric curve, the first due to free haemoglobin, migrating approximately with the β globulin, the second due to haptoglobin-bound haemoglobin migrating with the α_2 globulin.

INTRODUCTION.

This paper presents a technique for obtaining quantitative electrophoresis results from sera containing haemoglobin. The problem of the haemolysis of blood samples is of particular importance in the study of sera of primitive people as electrophoresis is a relatively sophisticated technique and it is usually necessary to transport the specimens from the field to a suitably equipped laboratory. Blood is usually collected (with no anticoagulant) into sterile vacuum venules which are packed in ice and transported by air. In spite of the best possible precautions we have found that 15 to 30 p.c. of such serum samples sent to us in different batches show significant haemolysis (>10 mg. haemoglobin per 100 ml. of serum).

Samples showing haemolysis cannot be discarded for two reasons. Firstly, it is often impossible to obtain further samples, and even when this is done they too are haemolysed; secondly, since the method of collection and transport is the same for each batch of serum, the haemolysis may be a result of increased red cell fragility amongst certain individuals in the sample. To discard the haemolysed sera would mean that the sample was no longer representative of the entire population.

[1] Part of the expenses of this investigation were defrayed by a grant from the National Health and Medical Research Council, Australia.

Materials and Methods.

Serum. Normal human serum was obtained from donors attending the Red Cross Blood Bank at the Alfred Hospital, Melbourne.

Haemoglobin. A pool of cells from the same donors was washed five times with six volumes of 0·14 M NaCl and haemolysed by freezing and thawing. The concentration of haemoglobin was estimated by reading the absorption at 540 mμ in a Beckman D.U. or a Hilger "Uvispek" spectrophotometer. A millimolar extinction co-efficient of 59·6 was used.

Haemolysed sera. The serum samples were divided into pairs. To one was added the desired amount of haemoglobin solution, to the other the same amount of distilled water. Both samples were dialysed for 24 hours against two changes of sodium veronal buffer, pH 8·6, I = 0·10. After dialysis the haemoglobin content of the first sample was determined in the spectrophotometer and the total protein content of both samples was determined refractometrically. The refractometer was based upon the design of Cecil and Ogston (1951) with the refinement of a photoelectric indicator. A value of 0·00184 was taken for the specific refractive increment. After allowing for the haemoglobin content of the "haemolysed" sample, both samples were adjusted to the same serum protein concentration by further dilution with the above buffer. The final serum protein concentration used in the electrophoresis was 1·50 gm. per 100 ml.

Electrophoresis apparatus.

A Perkin-Elmer model 38A Tiselius apparatus was used. The thermostat bath of this instrument was kept at 2° ± 0·02° C. with the aid of circulating refrigerant.

The instrument was fitted with a red light interferometric optical system of the Philpot-Cook (1948) type. The image of the vertical filament of a ribbon lamp was focused on a 0·02 × 30 mm. vertical slit. In front of this slit was placed a Bausch and Lomb 690 mμ second order interference filter. The remainder of the instrument's optical system was unaltered, except that the diagonal slit in front of the camera lens was removed. The flanged cell and its double slit mask supplied for the Moore and Opperman (1956) Rayleigh interferometric optical system was used. When it was desired to record schlieren diagrams, the system was converted to the Philpot-Svensson type by rotating the lamp and slit to the horizontal position and replacing the diagonal slit.

The fringe patterns were recorded on Kodak separation negative plates, which were developed in Kodak D.72 developer. Electrophoresis was carried out for 6,000 sec. and the plateholder described in the previous paper (Curtain, 1961) was used. The plates were evaluated with the aid of a photoelectric comparator. The design and operation of this instrument has been described elsewhere (Curtain, 1960). Gradient curves were obtained by plotting the reciprocal of each fringe spacing against mean distance of that fringe from the origin. This was carried out with the aid of a plotting table and computor (to be published).

RESULTS.

The distribution of haemoglobin in the electrophoretic pattern.

Fig. 1 shows the effect of increasing amounts of haemoglobin on the serum electrophoresis pattern. The haemoglobin is located in the β and α_2 globulin areas of the pattern. The amount of haemoglobin in the α_2 area varies significantly between the sera from different individuals. This haemoglobin is bound as the haptoglobin-haemoglobin complex (Nyman, 1959).

As the concentration of serum haptoglobin is extremely variable, ranging from 80 to 140 mg. p.c. in normal individuals, any formula for correcting electrophoresis patterns for the presence of haemoglobin must take this into account.

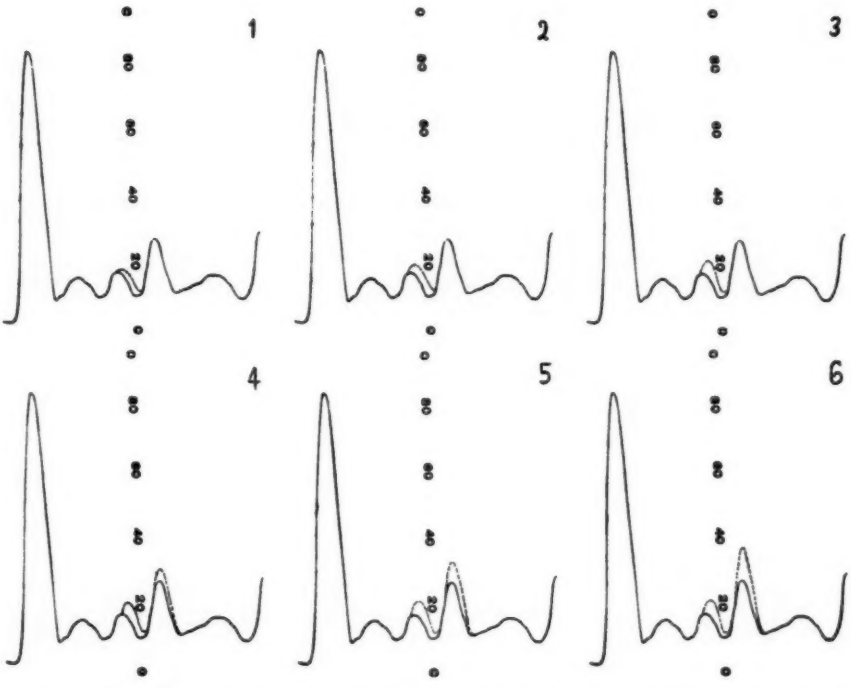

Fig. 1. The effect of increasing amounts of haemoglobin on the electrophoresis pattern of normal human serum. The solid line represents the pattern of the serum without added haemoglobin. The broken line in each figure represents the increment to the gradient curve caused by the addition of haemoglobin in the following amounts:

(1) 35 mg. p.c.
(2) 60 mg. p.c.
(3) 109 mg. p.c.
(4) 135 mg. p.c.
(5) 147 mg. p.c.
(6) 160 mg. p.c.

Electrophoresis was in each case carried out for 6,000 sec. at 18 v. cm.$^{-1}$ in sodium veronal buffer, pH 8·60, I = 0·10. The diagrams are chart recorder tracings obtained from the ascending pattern, as described in the text.

Direct estimation of haemoglobin in the electrophoresis cell.

If the inclined slit and cylindrical lens of the Philpot-Svensson optical system are removed an image of the electrophoresis cell is formed in the focal plane of the camera. If the cell contains a concentration gradient of a substance absorbing light of the wavelength of the source this gradient will be represented by a variation in the light intensity along the image. If the flanged Perkin-Elmer 2 cc. cell is used with the slit mask in position an image will also be obtained of the reference channel.

To estimate haemoglobin in the electrophoresis cell the red 690 mμ filter

was replaced by a green 546 mμ filter (normally used in the Moore and Opperman (1956) optical system). The source and slit were rotated to the horizontal position and the cylindrical lens moved out of the optical path. The electrophoresis cell was filled with veronal buffer (pH 8·60, I = 0·10) containing 500 mg. of haemoglobin per 100 ml. and the image of the cell photographed on a Kodak orthochromatic plate. The plate was exposed for 30 seconds and developed for 120 seconds in Kodak D.72 developer. Before fixing the plates were immersed for 30 seconds in a stop bath of 2 p.c. acetic acid. This procedure was repeated with the cell filled successively with solutions of 250, 125, 60, 30 and 15 mg. of haemoglobin per 100 ml. The optical density of the cell images on the plates was obtained by using the photoelectric comparator (Curtain, 1960) as a microdensitometer, although any commercial densitometer having the same resolution should serve equally well. The density of the image of the reference path was recorded for each plate as a check on the constancy of the exposure and processing. A calibration curve was constructed from the optical density reading and the known values of the haemoglobin solutions.

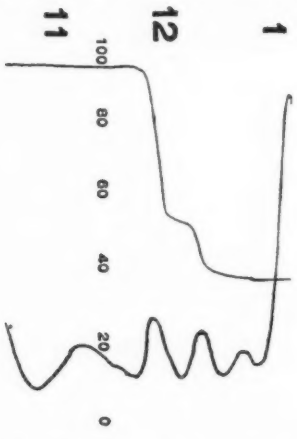

Fig. 2. The thin line is a densitometric curve obtained by scanning a photograph of the direct image of a Tiselius cell containing two optical density gradients, one due to free haemoglobin and the other due to the haptoglobin-haemoglobin complex.

The thick line is the serum ascending electrophoresis curve obtained from an interferometric pattern. Electrophoresis was carried out in veronal buffer, pH 8·60, I = 0·10, at 18 v. cm.$^{-1}$ for 6,000 sec.

In order to linearize the response of the microdensitometer, the calibration curve was photographed on a piece of 35 mm. film and inserted in a photo-electric function generator. Briefly, this device consists of a mirror galvanometer whose light source is masked by a photographic negative of the desired function. The galvanometer spot is focused on a photoelectric cell masked by a slit. With this arrangement the output current of the photocell can be made any desired function of the input current to the galvanometer. The function generator is connected between the D.C. amplifier and the chart recorder of the densitometer.

To measure the distribution of haemoglobin in the electrophoresis pattern, a haemolysed serum was submitted to electrophoresis in the usual manner. After the fringe pattern was obtained a direct photograph was taken of each limb of the electrophoresis cell under the standard conditions. The plates were scanned in the microdensitometer. A typical record is shown in Fig. 2. The gradient

curve derived from the fringe pattern is superimposed upon the record. The densitometer curve represents the haemoglobin concentration as a function of the distance along the cell, that is, it is the integral of the gradient curve due to haemoglobin. To correct the serum for haemolysis it is only necessary to measure the heights between the three steps and to convert these to haemoglobin concentrations in mg./100 ml. for the β and α_2 globulin regions of the pattern.

When comparison is made between the haemoglobin distributions so obtained and those found by superimposition of gradient curves before and after the addition of haemoglobin good agreement is found.

DISCUSSION.

The validity of this method as a means of correcting serum electrophoresis patterns for haemolysis depends on the assumption that free haemoglobin and the haemoglobin in the haptoglobin complex have the same extinction co-efficients at 546 mμ. We have been unable to find any reference to this in the literature but from our own observations it appears that the addition to a haemoglobin solution of serum of a normal haptoglobin content does not alter any portion of the absorption curve of haemoglobin, the extinction co-efficients at all wavelengths remaining the same.

It must be noted that this method provides a means of estimating the haptoglobin contents of sera which already contain excess haemoglobin under conditions where the method of electrophoretic titration (Nyman, 1959) could not be used.

It must be emphasised that the specialised densitometer and red-light interferometric optical system merely increase the convenience and accuracy. There is no reason why the principle could not be applied to electrophoresis instruments fitted with conventional Philpot-Svensson optics and an incandescent source. The two filters would still be needed and the direct cell image plates could be calibrated and read with any suitable commercial densitometer.

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REFERENCES.

Cecil, R., and Ogston, A. G. (1951): J. sci. Instrum., 28, p. 253.
Curtain, C. C. (1960): *Ibid.*, 37, p. 190.
Curtain, C. C. (1961): Aust. J. exp. Biol., in press.
Moore, D. H., and Opperman, K. O. (1956): Biochim. et biophys. Acta, 22, p. 136.
Nyman, M. (1959): Scand. J. lab. and clin. Invest. Suppl. 39.
Philpot, J. St. L., and Cook, J. H. (1948): Research, 1, p. 234.